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CONTENTS OF THIS ISSUE

The Effect of Glutamic Acid on *Datura tatula* L. Root Cultures 151
Donald I. French and *Melvin R. Gibson*

Steroidal Sapogenins XXXIX. Occurrence and Isolation of Gentrogenin and Correllogenin from *Dioscorea spiculiflora* .. 155
Monroe E. Wall, J. J. Willaman, Theodore Peristein, D. S. Correll, and *H. S. Gentry*

Bioassay of Aged Atropine Solutions 160
Edward J. Huyche

The Determination of Alkali Salts of Organic Acids by Residual Nonaqueous Titration 163
Martin I. Blake

A Comparative Study of the Local Anesthetic Potencies and Toxicities of *Beta*-Diethylaminoethyl Cumate and Procaine .. 167
Guilford C. Gross and *Elbert Voss*

Gitoside–A New Digitalis Glycoside 170
James E. Murphy

Analysis of Atropine and Its Hydrolytic Products by Filter Paper Chromatogrpahy 173
Herbert Schriftman and *Albert A. Kondritzer*

Glutarimides IV. N-Alkyl and N-Aryl Derivatives of 3-Phenylglutarimide and 3-Phenyl-2-Methylglutarimide 176
Billy G. Mallard and *Charles O. Wilson*

An Approach to Antacid Evaluation 179
Robert H. Schleif

The Nature of Scandol 183
Elias J. Corey, Stephen Proskow, and *Lloyd M. Parks*

Preliminary Investigation of the Fungistatic Properties and Toxicity of Certain Resorcinol Derivatives 185
Frank W. Zabransky and *Harold N. Wright*

Isonitrosoketones 188
George M. Steinberg and *James Bolger*

Determination of Protoveratrine II. Separation of Protoveratrines A and B from Associated Alkaloids 191
Joseph Levine and *Henry Fischbach*

Extraction of Morphine from Poppy Capsules and its Recovery by Ion Exchange 193
C. L. Mehltretter and *F. B. Weakley*

The Statistical Evaluation of a Biological Assay for Antiemetic Drugs 197
Joseph L. Ciminera, Clement A. Stone, and *Johannes Ipsen*

The Chemistry of Polyvinylpyrrolidone-Iodine 201
S. Siggia

A Note on the Preparation of Fe^{59} Tagged Exsiccated Ferrous Sulfate 204
E. Rosen and *I. B. Eisdorfer*

A Note on the Water Solubility of some N,N,-Dialkylamides 205
H. B. Kostenbauder and *T. Higuchi*

A Note on the Effect of Hyaluronidase on Sulfathiazole Renal Toxicity 206
Preston T. Farish and *B. B. Williams*

A Note on Fungicides: 2-Trichloromethanesulfenyl-6-Nitrosaccharin and Related Compounds 207
Glenn H. Hamor

A Note on a Simple Apparatus for Detecting Neurological Deficit in Rats and Mice 208
N. W. Dunham and *T. S. Miya*

Book Notices 209

Advertising ..... Cover pages 2, 3, 4, and I-IV

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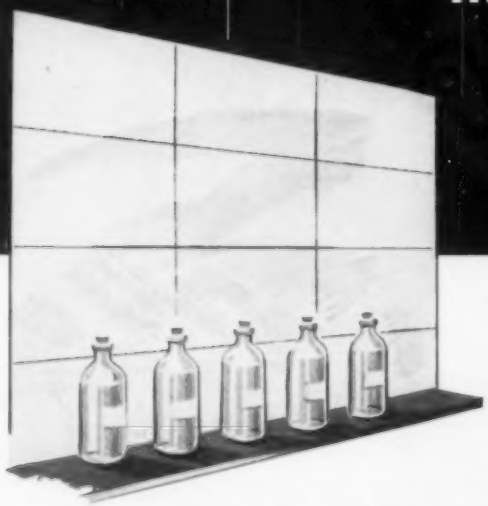

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VOLUME XLVI — *MARCH 1957* — NUMBER 3 — CONSECUTIVE NO. 6

The Effect of Glutamic Acid on *Datura tatula* L. Root Cultures*

By DONALD I. FRENCH and MELVIN R. GIBSON†

A study of the effect of glutamic acid in two concentrations on three-week-old root cultures of *Datura tatula* L. indicated a decrease in hyoscyamine and hyoscine content of test roots and a stimulation of growth and secondary root development.

THE RELATIONSHIPS between proteins, amino acids, and alkaloids have long been recognized and have been the basis for the majority of investigations, including this one, concerned with the biosynthesis of alkaloids. The work of Rogers (1) and Abelson (2) has suggested that glutamic acid may serve as a source of other related amino acids, namely, ornithine, arginine, and proline. The work of Leete, Marion, and Spenser (3, 4) with radioactive ornithine suggested that this substance may serve as a precursor to the tropine ring of hyoscyamine but not to the scopine ring of hyoscine in mature *Datura stramonium* L. Leete, *et al.*, suggest that scopine is possibly produced from a hypothetical hydroxyornithine which could conceivably be obtained from hydroxyproline. Two hypotheses have arisen with regard to the biosynthesis of hyoscyamine and hyoscine on the basis of evidence obtained in these and other investigations. The first, that tropine and scopine are synthesized by two different pathways in the plant, and the second, that the two are synthesized by the same, or similar, pathways but that hyoscine is formed only at a comparatively early stage in the development of the plant (5), and thus would not be influenced by the experimental procedures carried out on mature plants.

Working with this second hypothesis, Marion and Thomas (6) fed methionine with a C^{14}-labeled methyl group to mature *D. stramonium*. They found that in all cases of nitrogen methylation in plants studied thus far, methionine has always acted as the source of the methyl groups. From this it was logical to assume that the labeled methionine would perform the same function in *D. stramonium*. In examining their results they found that only the hyoscyamine was radioactive. This would seem to leave little doubt that Trautner's (5) original suggestion was valid, and hyoscine is not being produced in the mature plant.

Because glutamic acid may possibly serve as a common source of the amino acids which have been proposed as precursors to hyoscyamine and hyoscine, it was selected to be used in this investigation to study the synthesis of these alkaloids in three-week-old root cultures of *Datura tatula* L. And if the alkaloids are produced by the same, or

* Received July 30, 1956, from the School of Pharmacy, State College of Washington, Pullman.

Abstracted in part from a thesis submitted to the Graduate School of the State College of Washington by Donald I. French in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

† Professor of Pharmacognosy, State College of Washington, Pullman.

similar, pathways, but hyoscine only at an early stage of plant development as Trautner (5) suggests, one would still expect to observe an increase in both alkaloids because of the immature plant tissue being used.

EXPERIMENTAL

Growth and Processing of Root Cultures.—The *D. tatula* seeds used were field-grown and harvested in 1950 at the State College of Washington. They had been stored in glass bottles until used. The seeds were sterilized by shaking them with 3% hydrogen peroxide for fifteen minutes, then germinated aseptically on moist filter paper in sterile petri dishes wrapped in sterile aluminum foil. The germination usually required six days (at 26–27°). At the end of this period, using aseptic techniques, healthy roots between 1 and 1.5 cm. were excised and dropped into sterile culture flasks containing nutrient solution. The nutrient solution used for growth of control root cultures was White's nutrient (7). One excised root was placed in each flask, and the top of the flask sealed with a square of sterile aluminum foil to reduce evaporation of the media. The cultures were then placed in an incubator and grown in the dark at 26–27°. The root cultures were aseptically transferred weekly to new flasks with fresh, sterile nutrient. The roots were grown for three weeks. The water used in preparing the nutrient was distilled and then passed through a Barnstead Bantam Demineralizer, Model BD-1. Water was collected for use in the nutrient with less than 2 p. p. m. of ionized solids expressed in sodium chloride equivalents. Each culture flask, containing 50 cc. of nutrient, was stoppered with a cotton plug and sterilized in an autoclave before using. The cotton plugs were subsequently changed to foil seals. This operation and all transfers were done in a custom-built, stainless steel, sterile transfer chamber.

At the end of the three-week growth period, root cultures of approximately uniform size were removed from the flasks. The cultures were blotted to remove excess water, dipped momentarily into an ice-cooled bath of absolute ethanol, then quick frozen by dipping for fifteen seconds into petroleum ether cooled with solid carbon dioxide. The quick-frozen cultures were then dried in a histological freeze-dry apparatus (Model FD-11, Scientific Specialties Corp.). The drying required ninety-six hours, after which the dry roots were ground to a fine powder and stored in a desiccator. All cultures, control and experimental, were grown and processed in this manner with the exception that the experimental cultures were fed two different concentrations of L-glutamic acid.

Sanders and Burkholder (8) observed that L-glutamic acid present in a concentration of 60 p. p. m was available for growth and was not toxic to heart embryos of *Datura inoxia* Mill. and *D. stramonium* when nitrate was also present. More recent work by Paris, Rietsma, Satina, and Blakeslee (9) showed that neither L- nor D-glutamic acid in concentrations of 400 or 80 p. p.m. caused an increase in growth of excised *D. stramonium* embryos. On the basis of these investigations, it was decided that the two experimental concentrations of L-glutamic acid to be used in this work would be 20 p. p. m. and 60 p. p. m. Typical cultures grown under control and experimental conditions are shown in Figs. 1, 2, and 3.

Extraction and Separation of Alkaloids.—The alkaloids were extracted and separated from the crude drug in the following manner. Twenty-five milligram samples of the powder were moistened with eight drops of a 1:1 solution of 10% ammonia and 95% ethanol, allowed to stand one hour, and extracted with 25 cc. of chloroform. The extract was allowed to drip slowly onto a strip of Whatman No. 1 chromatograph paper where it was evaporated by a stream of air. In this manner all of the

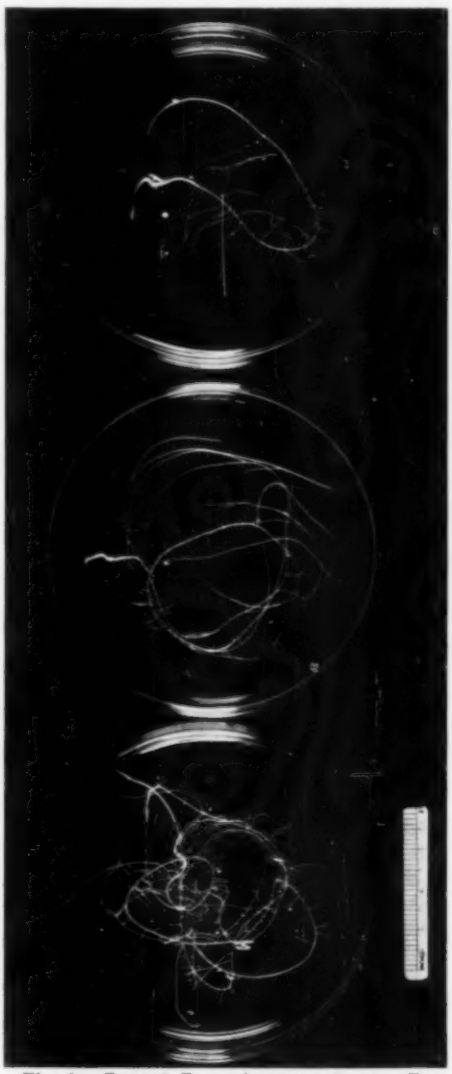

Fig. 1.—Control *D. tatula* root cultures. Top, one-week growth; middle, two-week growth; bottom, three-week growth.

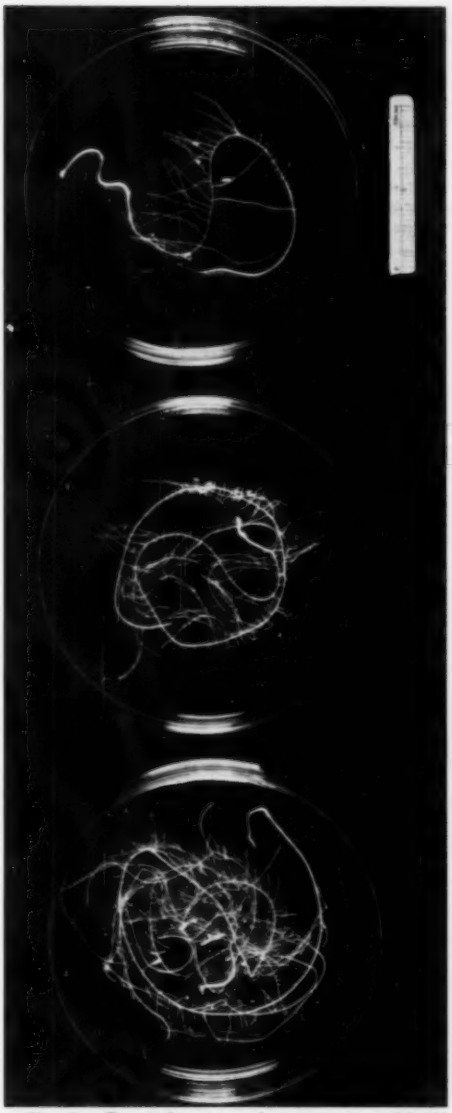

Fig. 2.—*D. tatula* root cultures grown with 20 p. p. m. L- glutamic acid. Top, one-week growth; middle, two-week growth; bottom, three-week growth.

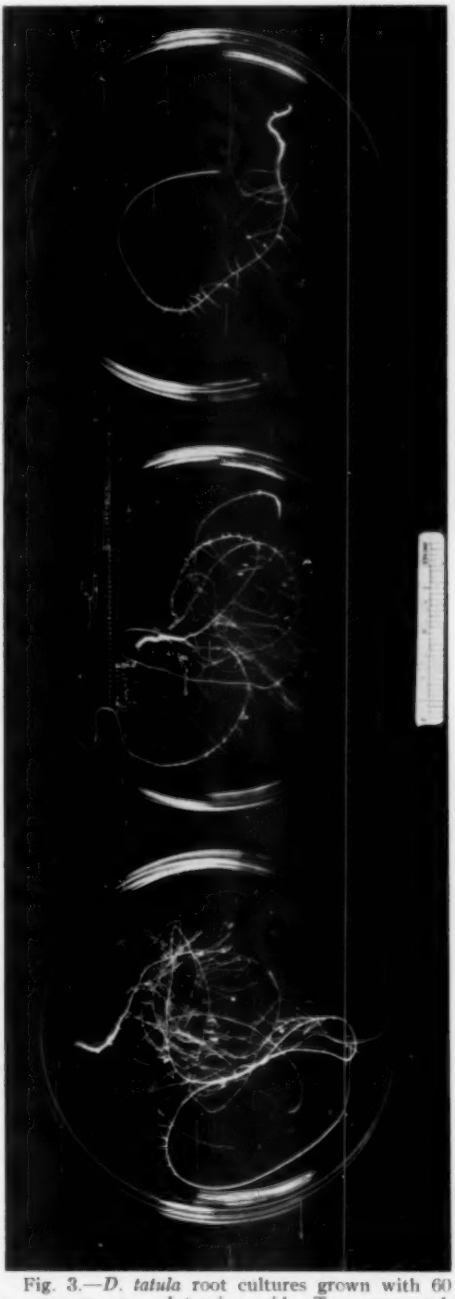

Fig. 3.—*D. tatula* root cultures grown with 60 p. p. m. L- glutamic acid. Top, one-week growth; middle, two-week growth; bottom, three-week growth.

extract was placed on a small spot of the paper. The paper strips, spotted in this manner, were placed in a small, airtight, glass chromatograph chamber and developed in an ascending manner for ten hours. The developing solvent consisted of 15 cc. of concentrated hydrochloric acid, 100 cc. of *n*-butanol, and the resulting solution saturated with distilled water.

At the end of each development period, the solvent was removed from the chamber; one of the four chromatograms was removed and air dried. The other strips were allowed to remain in the saturated atmosphere of the chamber until needed. The

one dried chromatogram was passed through a bath of Dragendorff's reagent (10) and served as a control strip on which the alkaloid spots were located. The positions of hyoscine and hyoscyamine on the chromatograms of the extract were originally identified by comparing the alkaloid spots with those on chromatograms prepared from the pure alkaloids and a mixture of the pure alkaloids.

With the alkaloid bands identified, and using the control strip as a guide, the sections of the chromatograms containing hyoscyamine and hyoscine were removed and the alkaloids eluted using a solvent composed of 3% hydrochloric acid in 10% ethanol. The techniques of spotting and elution of the paper strips comprise another paper by the authors (11). The eluates were collected in evaporating dishes, evaporated to dryness under a stream of air, and the residues assayed using the Vitali-Morin reaction as reported by Colby and Beal (12). The color which developed was measured spectrophotometrically in a Beckman DU Spectrophotometer at 560 mμ.

RESULTS

A series of ten assays was carried out on each alkaloid for each of the three drug powders. The readings, percentage of transmittance, for each series were converted into milligrams of alkaloid in the usual manner using the Bouguer-Beer relationships as expressed by Mellon (13). The results of the alkaloid assays are given in Table I.

TABLE I.—RESULTS OF ALKALOID ASSAYS

	Control		20 p. p. m. L-Glutamic Acid		60 p. p. m. L-Glutamic Acid	
Assay	Hyoscyamine	Hyoscine	Hyoscyamine	Hyoscine	Hyoscyamine	Hyoscine
1	0.0823 mg.	0.0236 mg.	0.0734 mg.	0.0197 mg.	0.0643 mg.	0.0152 mg.
2	0.0796	0.0199	0.0755	0.0199	0.0617	0.0145
3	0.0807	0.0208	0.0744	0.0216	0.0638	0.0147
4	0.0828	0.0236	0.0729	0.0201	0.0655	0.0156
5	0.0839	0.0206	0.0729	0.0187	0.0634	0.0149
6	0.0810	0.0218	0.0704	0.0208	0.0622	0.0160
7	0.0796	0.0226	0.0757	0.0191	0.0601	0.0156
8	0.0825	0.0228	0.0712	0.0189	0.0615	0.0160
9	0.0812	0.0206	0.0742	0.0181	0.0629	0.0170
10	0.0770	0.0220	0.0737	0.0179	0.0631	0.0158
Mean	0.0811 ± 0.0018	0.0218 ± 0.0012	0.0734 ± 0.0015	0.0195 ± 0.001	0.0628 ± 0.0014	0.0155 ± 0.00067

The results recorded indicate that glutamic acid did not stimulate the production of alkaloids in *D. tatula* root cultures, but significant decreases in alkaloid content were observed with increasing glutamic acid concentrations. These decreases were found to be statistically significant at the 99.73% confidence level. Average dry weight determinations showed that the weight of the control roots averaged 12.3 mg., the roots grown in 20 p. p. m. glutamic acid, 17.8 mg., and those grown in 60 p. p. m., 15.5 mg. The observation was also made that apparently glutamic acid induced or enhanced the formation of secondary roots; this difference in secondary root growth is shown in Figs. 1, 2, and 3.

DISCUSSION

The hypothesis that glutamic acid possibly serves as an indirect precursor to hyoscyamine and hyoscine, as expressed in the first part of this paper, is not supported by the results obtained under the conditions of this experiment.

It appears that the nitrogenous constituents of plants are in a dynamic state which might be described as complex and variable equilibria. The general picture of alkaloid formation is one of a synthesis running parallel with that of the proteins; the conditions favoring the one also favoring the other and both in a rather unbalanced competition for the available nitrogen and carbohydrate supplies of the plant. That the alkaloids are the unsuccessful competitor has been demonstrated by James (14) who found that 95% of the total nitrogen in young *Atropa belladonna* L. leaves was in the proteins and only 0.3–0.9% in the alkaloids. Certain amino acids of either primary or secondary formation are potential alkaloid precursors; however, in three-week-old root cultures, such as used in this investigation, there would be very little protein degradation, hence, the majority of the amino acids would arise by primary formation.

The fact that the root cultures were young, actively, and rapidly growing tissues would suggest that protein synthesis was being carried out at a high rate. This synthesis would place great demands on the amino acid production of the tissue particularly since the only amino acid supplied by White's nutrient (7) is glycine. By feeding the root cultures with White's nutrient supplemented with glutamic acid, the mechanisms of protein synthesis would have a readily available source of glutamic acid itself and possibly other amino acids which might arise from it in the manner described by Abelson (2). This would seem to have the effect of enhancing protein synthesis, possibly at some expense to the alkaloids. The increased growth observed in those root cultures fed glutamic acid would lend support to the belief that there was an increase in protein synthesis. The explanation becomes even more feasible in light of the work of James (14) who has repeatedly observed slight reductions in the alkaloid percentage of young belladonna leaves when protein synthesis was stimulated by potassium.

The concentration of 20 p. p. m. glutamic acid gave a greater increase in growth than the concentration of 60 p. p. m., indicating that the former concentration was closer to an optimum for growth stimulation; the latter possibly provided an excess of glutamic acid to the cultures. If glutamic acid serves as an alkaloid precursor, one would expect this excess to become evident by an increase in alka-

loids in those root cultures fed 60 p. p. m. glutamic acid; however, it was observed that these cultures contained *less* alkaloids than the cultures fed 20 p. p. m. glutamic acid. This indicated that the glutamic acid was being channeled into some pathway other than that concerned with alkaloid synthesis.

SUMMARY

L-Glutamic acid in the concentrations and under the conditions described in this investigation, produces the following effects in three-week-old root cultures of *D. tatula:*

1. An increase in growth results, as evidenced by dry weight determinations; the greatest increase is obtained with a concentration of 20 p. p. m. glutamic acid.

2. There is an increase in the number of secondary or side roots.

3. There is a slight, but statistically significant, decrease in alkaloid content of the roots when increasing concentrations of glutamic acid are used.

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Steroidal Sapogenins XXXIX.*

Occurrence and Isolation of Gentrogenin and Correllogenin from *Dioscorea spiculiflora*

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Two new isomeric 12-ketonic sapogenins have been isolated from tubers of *Dioscorea spiculiflora,* obtained in Chiapas province, Mexico. The new sapogenins, called gentrogenin and corellogenin, constitute 20 to 55 per cent of the total sapogenin content which ranges from 2.0 to 5.5 per cent, moisture free basis. Methods for the analysis and isolation of gentrogenin and correllogenin are presented in detail. The more important physical constants including melting point, optical rotation, and infrared spectra are recorded.

AFTER KENDALL AND HENCH first announced the dramatic antiarthritic effects of cortisone in 1948, teams of plant explorers and chemists from various laboratories made great efforts to find suitable plant steroid precursors for this valuable drug. In 1947, Marker and Lopez had reported the isolation of botogenin from *Dioscorea mexicana* tubers (1). Since botogenin was reported to have both the 12 ketone and the $\Delta^{5(6)}$ unsaturated bond, it would have been a very desirable precursor. In subsequent years it has been the object of assiduous searches both by our group and teams from other laboratories, with uniformly negative results. Recently we announced the discovery of two new sapogenins, gentrogenin and correllogenin (2, 3). Gentrogenin and correllogenin have the same structure as those assigned to botogenin and neobotogenin,

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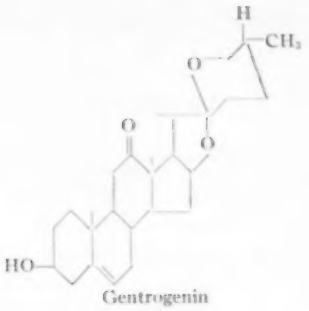
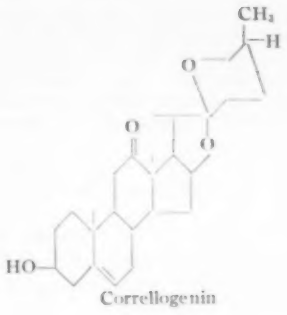
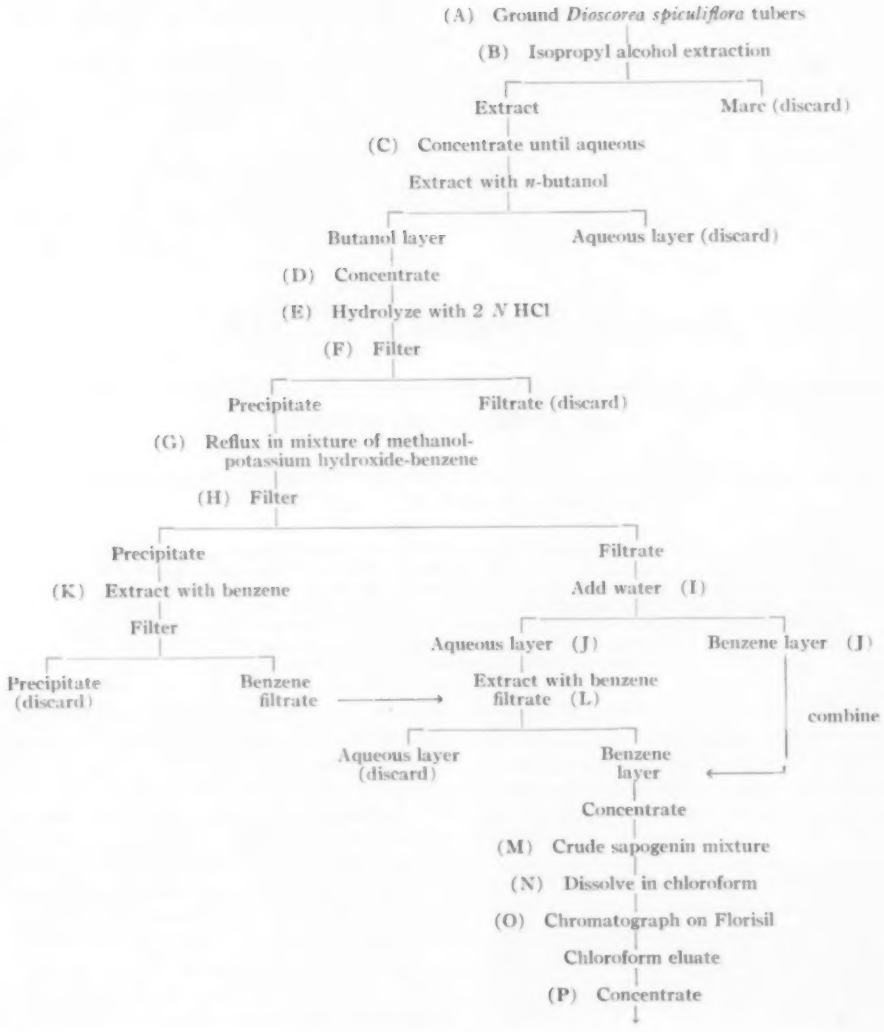


Gentrogenin



Correllogenin



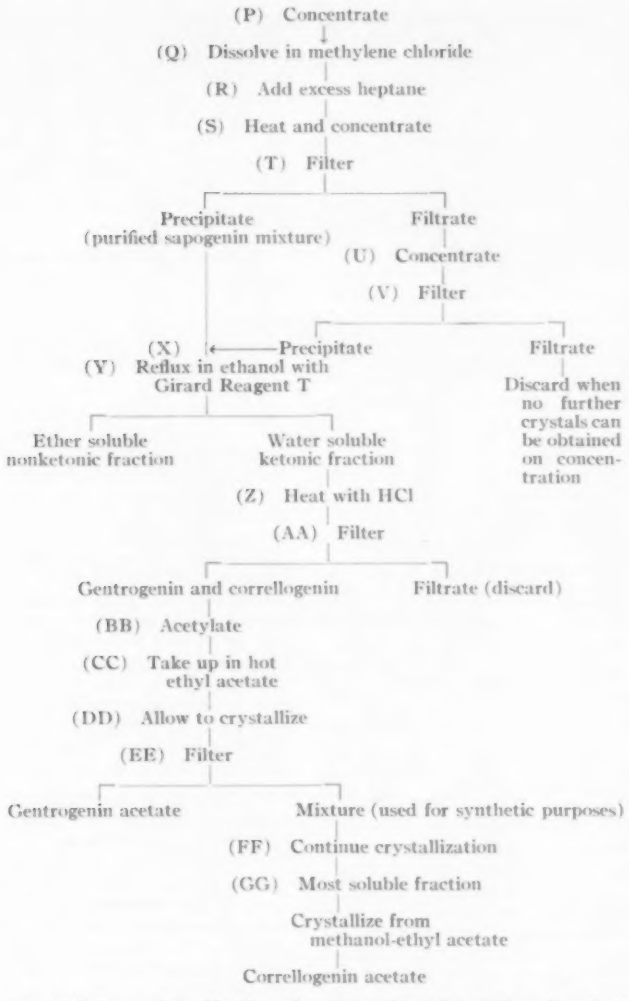


Fig. 1.—Flowsheet for isolation and purification of gentrogenin and correllogenin. Letters on flowsheet correspond to similar letters in text.

respectively (4). However, the properties of the new sapogenins differ greatly (2, 3) from those reported by Marker (4). It is the object of this paper to report additional information on the occurrence and isolation of gentrogenin and correllogenin.

OCCURRENCE

To date gentrogenin and correllogenin have been found only in the tubers of *Dioscorea spiculiflora*. This species has been found in Chiapas province of Mexico near the Guatemalan border. While it may occur in other regions of Mexico or Central America, results of extensive surveys in our laboratory indicate that it cannot be of common occurrence in other areas (5–8). Table I gives the data on 12 samples of *D. spiculiflora*.

The total sapogenin content varied from 2.0–5.5%, moisture free basis, the average being 3.5%. The ketonic fraction varied from 20–55% of the total sapogenin, the average being approximately 40%. The nonketonic fractions consisted of diosgenin and yamogenin, the 12 desoxyanalogs of gentrogenin and correllogenin, respectively. Gentrogenin and diosgenin were predominant in the ketonic and nonketonic fractions, respectively. Thus the sapogenins in *D. spiculiflora* consist invariably of a mixture of

12 desoxy- and 12 keto-sapogenins with the 20α, 25D side chain predominant and a minor proportion of the respective 20α, 25L isomers. A review of side chain stereochemistry and nomenclature can be found in a recent paper (9).

Of interest is the fact that configuration of the side chain in *Dioscorea* seems to be species specific. Thus diosgenin (25D) is the sole constituent of the commercially important *D. composita;* in the equally important *D. floribunda* a mixture of diosgenin and yamogenin (25L) is found; similar side chain mixtures occur in *D. spiculiflora;* and in *D. bartletti* yamogenin is sometimes the sole constituent.

TABLE I.—OCCURRENCE OF GENTROGENIN AND CORRELOGENIN IN DIOSCOREA SPICULIFLORA

EURB No.	Date	Location (Chiapas)	Total Sapogenin % MFB	Ketonic Fraction %
3134	10/52	Cerro Juarez	3.2	30
3140	10/52	Tuxtla Gutierrez	2.0	25
3513	6/53	Tuxtla Gutierrez	3.0	20
5317	3/55	Cristobal	3.6	35
5767	12/55	Chiapa	3.7	30
5768	"	Tuxtla Gutierrez	5.5	50
5769	"	Tuxtla Gutierrez	4.7	55
5770	"	Oaxaca	3.8	40
5771	"	Oaxaca	3.0	35
5772	"	Oaxaca	2.0	45
5773	"	Oaxaca	5.0	55
5792	"	Tuxtla Gutierrez	2.9	35

ISOLATION OF GENTROGENIN AND CORRELLOGENIN

Figure 1 gives an outline of the processes used.

Extraction of Saponins.—The following procedure was typical. *Dioscorea spiculiflora* tubers, 27.6 Kg., 68% moisture, were ground to pass a one-inch screen on a Ball and Jewell[1] cutter (A). The ground tubers were immediately extracted with 60 liters of 95% isopropyl alcohol in a 50-gallon steam-jacketed kettle equipped with agitator and reflux condenser. After refluxing one hour, the mixture was cooled and the alcohol run off. The extraction was repeated twice (B). The marc was discarded and the extracts combined and concentrated at atmospheric pressure to a volume of 18.2 liters (C).

Purification of Saponins.—The essentially aqueous suspension thus obtained was adjusted to pH 4.0 with concentrated hydrochloric acid and 1.0 Kg. of technical sodium chloride added. These additions repress the solubility of the saponin in the aqueous phase. The aqueous suspension was extracted seven times with *n*-butanol using 3 liters for each extraction. The aqueous fraction was discarded and the combined butanol extracts washed once with one-fourth volume of 5% sodium chloride solution. The sodium chloride solution was extracted with an equal volume of butanol and then discarded, the butanol being added to the original butanol solution. For large-scale extraction we used a glass-lined kettle provided with an agitator and a sight glass. Alternatively large separatory funnels can be used dividing the original aqueous extract in batches of appropriate volumes.

The combined butanol extracts (24.6 liters) containing the purified *Dioscorea* saponins were placed in a 10-gallon still and 13.8 liters of water added. The constant boiling water-butanol mixture was distilled until all the butanol had been removed, leaving 7.4 liters of an aqueous suspension of purified saponins (D).

Hydrolysis of Saponins.—To the above aqueous suspension was added 2.5 liters of ethanol and 2.5 liters of 9.9 *N* hydrochloric acid making the final acidity 2 *N*. The acidic solution was refluxed 4 hours (E), cooled, and filtered (F). The filtrate was discarded; the precipitate was washed well with water.

Alkaline Purification of Sapogenins.—The precipitate thus obtained, consisting of a mixture of sapogenins, resins, and tars, was refluxed 15 minutes in a mixture of 2 liters of methanol containing 400 Gm. of potassium hydroxide and 6 liters of benzene (G). After cooling the solution was filtered (H) and the precipitate saved. To the filtrate was added 2 liters of water (I) and the aqueous layer was withdrawn. Both the benzene and aqueous fractions were saved (J). The precipitate, described above, was extracted twice with 4 liters of benzene each time (K) and then discarded. The benzene was used to re-extract the above aqueous layer (L), the latter then being discarded. All the benzene extracts were then combined and the solvent distilled, leaving 541 Gm. of crude sapogenin mixture (M). The alkaline treatment removes much tar and resin of acid or phenolic nature.

Purification of Sapogenins.—The crude sapogenins were dissolved in 5 liters of technical chloroform (N) and passed through 1.0 Kg. of Florisil (O) in a 4-inch diameter column. The adsorbent was then eluted with 15 liters of chloroform and the combined eluates concentrated to dryness (P). The residue was dissolved in 2.5 liters of methylene chloride (Q). This solution was mixed with 7.5 liters of heptane (R) and heated. The solution was then concentrated to 6.0 liters and allowed to stand (S). The crystalline precipitate which formed was filtered and washed with cold heptane (T). The filtrate was concentrated to 3.0 liters (U), the precipitate again filtered (V), and this process was repeated until only a syrup could be obtained. The yield of crystallized sapogenin mixture was 321 Gm. or 3.7% of the tubers, moisture free basis (X). Infrared analysis showed that 30% of the sapogenin mixture was ketonic with gentrogenin predominant.

Separation of Ketonic Fraction.—The purified sapogenins were dissolved in 3 liters of hot, absolute ethanol containing 300 ml. of glacial acetic acid. In order to separate the ketonic and nonketonic fractions 180 Gm. of Girard Reagent T was added to this solution and the mixture refluxed one-half hour (Y). The solution was cooled and neutralized with 3 liters of ice cold saturated sodium carbonate solution containing 200 Gm. of sodium carbonate. This mixture was extracted six times with ether, using 2 liters each time. The combined ethereal fractions were washed six times using 2 liters of water

[1] The use of trade names does not imply recommendation over similar products which could perform the same function.

each time. In this manner the ether-soluble nonketonic fractions were separated from the water-soluble Girard T derivatives of the ketonic sapogenins. In order to hydrolyze the Girard T derivatives and thus obtain the original sapogenins all the aqueous fractions plus the sodium carbonate solution were combined and acidified to pH 1.0 with concentrated hydrochloric acid (Z). The mixture was heated on the steam bath one hour, cooled and filtered (AA). The precipitate was washed well with water and all the filtrates discarded. The precipitate was dried *in vacuo* at 80° for 10 hours, and weighed 92 Gm. Infrared analysis indicated that it was 100% ketonic sapogenin.

Separation of Gentrogenin and Correllogenin.—The above ketonic fraction was converted to the acetate by refluxing 2 hours with 450 ml. of acetic anhydride (BB). The acetic anhydride was removed by distillation *in vacuo* and the last traces removed by drying in a vacuum oven at 80°. The mixture was refluxed with 1.5 liters of ethyl acetate (CC) and allowed to stand at room temperature (DD). The long rectangular crystals which formed were filtered (EE). This product was pure gentrogenin acetate, yield 25.0 Gm. The mother liquors were concentrated, allowed to crystallize, and the process repeated until no more crystals were obtained (FF). Most of these fractions were mixtures of gentrogenin and correllogenin acetates. They are useful for synthetic purposes as they become identical on side chain degradation. The last and most soluble fractions were enriched in correllogenin acetate. Repeated crystallization from a 1-1 mixture of methanol-ethyl acetate gave 0.5 Gm. of pure correllogenin acetate (GG).

Physical Properties.—The physical properties of the new sapogenins are as follows: gentrogenin, m. p. 215–216°, $[\alpha]_D^{25}$ −57°; gentrogenin acetate, m. p. 227°, $[\alpha]_D^{25}$ −56°; correllogenin, m. p. 209–210°, $[\alpha]_D^{25}$ −69°; correllogenin acetate, m. p. 213–214°, $[\alpha]_D^{25}$ −60°.

Infrared Spectra.—One of the most useful properties for the detection of correllogenin and gentrogenin in mixtures is the infrared spectra of these compounds. Figure 2 gives the infrared spectra of gentrogenin acetate (top) and correllogenin acetate (bottom). The strong band at 1713 cm.$^{-1}$ slightly stronger than the 1737 cm.$^{-1}$ acetate band is characteristic of gentrogenin and correllogenin acetates and is assigned to 12 ketones in steroids (10). This band is of approximately equal intensity in both compounds and hence is particularly useful in calculating the percentage of ketonic sapogenins found in mixtures with nonketonic sapogenins. Several bands which are useful in determining the percentage of correllogenin or gentrogenin in mixtures of the two are found near 900 and 920 cm.$^{-1}$ In the case of correllogenin and similar 25L sapogenins the 920 band is strong and the 900 band weak. The reverse is true in the case of gentrogenin and other 25D sapogenins. It is best to make a series of known mixtures and then determine the absorptivities at these two regions (11). Then the percentages of correllogenin or gentrogenin may be approximated.

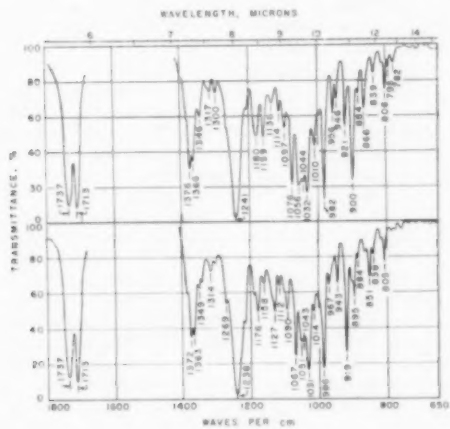


Figure 2.

SUMMARY

1. Gentrogenin and correllogenin, isomeric 12-ketonic sapogenins, have been isolated from tubers of *D. spiculiflora* with yields varying from 2.0 to 5.5 per cent total sapogenin and the ketonic fraction ranging from 20 to 55 per cent of the total sapogenin.

2. Gentrogenin and correllogenin were isolated by extraction and purification of their saponins followed by acid hydrolysis to the crude sapogenin. The sapogenins were purified by alkaline treatment followed by chromatography and crystallization. Gentrogenin and correllogenin were separated from nonketonic sapogenins (diosgenin and yamogenin) by treatment with Girard's reagent T and were then acetylated. Crystallization from ethyl acetate gave the insoluble gentrogenin acetate. Correllogenin in pure form was obtained from the soluble mother liquors. The more important physical constants including melting point, optical rotation, and infrared spectrum are presented.

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Bioassay of Aged Atropine Solutions*

By EDWARD J. HUYCKE†

The availability of aqueous solutions of atropine salts which had been stored for up to sixty-one months prompted the investigation of the effect of aging upon deterioration of physiologic activity of these solutions. Three methods of bioassay, mouse pupil, cat eye, and human eye, were adapted to quantitate the deterioration. Methods of bioassay agreed and showed deterioration of physiologic activity of about 35 per cent in samples five years old. The mouse pupil method is a better laboratory tool for atropine bioassay than either of the other two methods. The effect of tropine upon the mouse eye was studied.

THE USUAL PRACTICE of storing atropine salts in the solid form has not necessitated much concern over the possibility of deterioration of aqueous solutions of atropine stored for extended periods of time. Ophthalmologic drops are virtually the only aqueous form of atropine kept in clinical stocks, and these buffered solutions carry a stipulation for monthly renewal. Hydrolysis of the ester linkage between tropine and tropic acid is the principal form of chemical deterioration (1).

Availability to this laboratory of aged, aqueous solutions of atropine sulfate and atropine tartrate prompted the investigation of deterioration of physiologic activity in these solutions. Three methods of bioassay, mouse pupil, cat eye, and human eye, were adapted to quantitate the deterioration of physiologic activity observed in the aged solutions.

EXPERIMENTAL

Samples of commercially packaged atropine sulfate and atropine tartrate for injection were tested after having been stored under warehouse conditions. All samples were protected from light. The age of each sample indicates the time in months from the date of manufacture to the date of assay. Atropine sulfate samples contained 0.8 Gm. % NaCl, 0.13 Gm. % sodium acetate, and 48 mg. % glacial acetic acid; atropine tartrate samples contained 2.4 Gm. % glycerin and 0.4 Gm. % phenol. Potency at time of manufacture met specifications of 100 ± 5% of labeled amount.

Bioassay.—Bioassay was done by pupillary dilation measurements utilizing three species: mouse, cat, and man.

Dosage figures are calculated as levels of atropine base throughout the paper. Dilutions were made with distilled water. Standards were made daily from atropine sulfate powder, U. S. P. AS represents atropine sulfate and AT represents atropine tartrate throughout. Tropine was prepared by the method of Findlay (2).

Mouse Pupil Method (3).—Atropine was bioassayed for relative potency using the graded dose-response of the mouse pupil. Aged and standard solutions were compared with one another in each assay. A graphic representation of one such bioassay is given in Fig. 1.

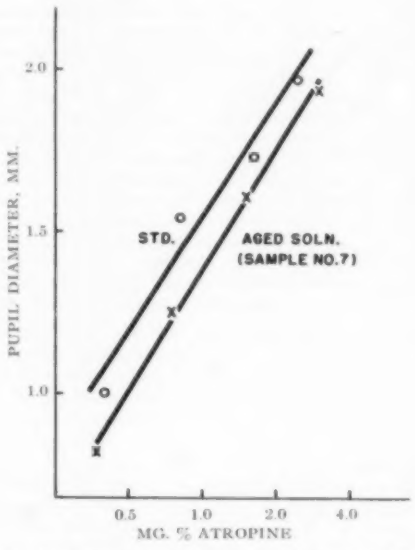

Fig. 1.—Mouse pupil bioassay of aged solution compared with standard.

Regular male laboratory mice weighing 15 to 20 Gm. were used. The mice were fed Hunt Club Diet for Laboratory Animals. Ninety mice normally comprised each group tested. Injections were given no oftener than every other day, and no mouse received more than three injections.

Each mouse was given an intraperitoneal injection of 0.2 ml. of appropriately diluted atropine solution. Four dilutions of standard were normally used: 0.4, 0.8, 1.6, and 2.4 mg. %. Appropriate dilutions were made of the aged solutions until their physiologic response resided in approximately the same range as standard. These solutions had the following pH values: standard, pH 5.5; aged solutions, pH 4.5 to 5.25. All aged atropine solutions were clear and colorless at the time of assay.

Ten mice were used at each dilution level of standard or aged solution. Ten uninjected mice served as controls. Readings on injected mice

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equal to, or less than the maximal reading obtained from the controls were discarded as representing faulty injections (into the bowel or bladder). Prior to injection, no mouse having a dilation greater than the maximal control dilation was used in the test. Intraperitoneal injections of distilled water, normal saline, and 0.4% phenol solution did not alter control values.

A standard against standard assay was run to give an estimate of intrinsic error. Portions of sample no. 6 were boiled for twelve hours and heated at 86° for 172 hours; these portions were similarly bioassayed to study heat accelerated hydrolysis of atropine tartrate at pH 5.12. In one assay the mice received an intrathoracic injection of 0.05 ml. of 4.0% tropine hydrochloride thirty seconds before the regular intraperitoneal injection of atropine. Another assay was done with equimolar tropine mixed with atropine at the time of injection.

Fifteen to eighteen minutes following injection each mouse was hand held on the stage of a binocular dissecting microscope. A 100-watt microscope lamp with condensing lens was placed ten centimeters from the head of the mouse to give standard lighting conditions. Pupil diameter was measured by means of an ocular micrometer which gave 1 mm. = 8.88 scale divisions; the microscope magnified 12.6 times.

TABLE I.—AGE AND RELATIVE POTENCY OF SAMPLES—MOUSE PUPIL METHOD

Sample No.	Salt and Age in Months	Relative Potency, %	95% Confidence Limits, %
1	AS, 61[a]	67.0	58.8–76.5
2	AS, 29[b]	60.9	50.8–73.2
3	AS, 29[b]	90.9	80.1–103.1
4	AT, 52[c]	66.6	56.1–78.7
5	AT, 51[c]	65.3	53.9–78.2
6	AT, 38[c]	73.7	62.6–86.2
7	AT, 15[c]	76.8	69.1–85.0
8	AT, 12[c]	90.3	78.6–104.1
9	Standard, range		94.5–106.0
10	Sample No. 6, pH 5.12 boiled 12 hr.	87.0	75.0–101.6
11	Sample No. 6, pH 5.12 heated at 86° for 172 hr.	76.6	70.4–82.6
12	Standard plus equimolar tropine	93.1	84.4–102.5
13	Standard with prior IT tropine	91.0	80.1–103.3

[a] Stored in glass vials. [b] Different lot numbers stored in plastic vials oversealed with metal foil. [c] Stored in metal containers.

TABLE II.—BIOASSAY—CAT EYE METHOD

	Standard —Atropine Base—		AS, 29 months —(Sample No. 2)[a]—		AT, 15 months —(Sample No. 7)[b]—	
	0.75 mg. %	0.5 mg. %	1.0 mg. %	1.25 mg. %	0.75 mg. %	0.875 mg. %
Cats with discernible pupillary dilation	14	0	1	6	0	2
Cats without discernible pupillary dilation	0	14	8	0	1	1

[a] AS, 29 months (Sample No. 2): (0.75 mg. %/1.25 mg. %) × 100 = 60% relative potency; range of accuracy: 50%–74%.
[b] AT, 15 months (Sample No. 7): (0.75 mg. %/0.875 mg. %) × 100 = 86% relative potency; range of accuracy: 69%–100%.

Statistically the data gave significantly linear regressions (log dose-response), $p < 0.05$. The slopes of the standard and aged atropine curves were not significantly different. The data were then analyzed by Fieller's method (4) to give relative potency and 95% confidence limits.[1] Duplicates on sample nos. 1, 3, 4, 5, 6, and 7 fell within these limits. (Table I.)

Cat Eye Method (5).—Adult cats in good physical condition, weighing over 1.2 Kg. were used. During instillations of solutions and pupillary measurements the cats were placed in restraining boxes. Prior to instillation of solutions a 100-watt frosted bulb was placed one foot from the cat's head, and measurements were made to insure equality of pupils. Then 0.05 ml. of atropine solution was dropped in a lower conjunctival sac, and the lower lid was manually opened and closed for thirty seconds while mild pressure was exerted over the inner canthus.

Four hours following instillation, measurements were made in front of the same light source. Measurements consisted of determining whether the pupil in which the instillation was made was discernibly larger than the control pupil. The cats were used no oftener than forty-eight hours after prior use.

Only a limited number of bioassays were run by this method because of difficulty in establishing narrow confidence limits with small numbers of cats. Data and results are presented in Table II.

Human Eye Method.[2]—White adults, ranging in age from twenty to thirty-five years, were used. Approximately equal numbers of males and females were included. Subjects were free of ocular inflammations, neoplasia and congenital deformities. Pigment of the irides ranged from light blue to dark brown.

Standard atropine solutions were made to 2.5

[1] Grateful acknowledgment is given to Dr. Max Halperin, Division of Biologic Standards, National Institutes of Health, Bethesda, Md., for deriving the prototypic analysis of these data.

[2] This work was done by Lt. Col. Jack W. Passmore, MC, Chief, Ocular Research Unit, Walter Reed Army Medical Center, Washington, D. C.

mg. % and 1.667 mg. %; aged atropine solutions were diluted to 2.5 mg. %.

Solutions were instilled in lower conjunctival sacs with a 0.025-ml. micropipet. Mild pressure was then exerted over the inner canthus for approximately thirty seconds.

Readings of pupil size were taken before and two hours after instillation. Uniform moderate lighting prevailed during measurements. Readings were taken by means of a pupillometer designed and fabricated by the Naval Gun Factory, Washington, D. C. At the dosage levels used cycloplegia was minimal or absent. Mydriatic and cycloplegic effects, if present, were maximal at two hours and gradually disappeared in four to six hours following instillation. In aged solutions with phenol as a preservative, instillation resulted in placing 12.5 μg. phenol in the conjunctival sac; this amount caused no pain or frank inflammation. Data and results are presented in Table III.

TABLE III.—BIOASSAY BY HUMAN EYE METHOD

AS, 29 months (Sample No. 2)	
Dilation Resulting From	Dilation Resulting From
2.5 mg. % standard >	2.5 mg. % aged solution (in 8 of 9 subjects)
2.5 mg. % standard =	2.5 mg. % aged solution (in 1 of 9 subjects)
1.667 mg. % standard >	2.5 mg. % aged solution (in 5 of 8 subjects)
1.667 mg. % standard <	2.5 mg. % aged solution (in 3 of 8 subjects)
(1.667 mg. %/2.5 mg. %) × 100 ≈ 66.7% relative potency	

With a uniform dose, blue irides reacted with more mydriasis than darker irides. Hence comparisons of standards in one eye were made against aged solution in the other eye of the same person. This method was developed in conjunction with the Ocular Research Unit, Walter Reed Army Medical Center, Washington, D. C., to test conformation of relative potencies obtained by animal and human bioassays and to study the feasibility of using humans as bioassay subjects for mydriatic drugs.

DISCUSSION

The need for bioassay has steadily decreased with advances in synthetic and quantitative pharmaceutical chemistry. However, bioassay remains as an indispensable pharmacologic tool in estimating and studying biologic response to plant and animal extracts that as yet do not have exact chemical syntheses or analyses. Atropine may be determined by chemical methods (6), but the bioassay of atropine is still not obsolete, particularly in estimating atropine in tissues, serums, or in the presence of chemical contaminants.

The mouse pupil assay is rapid, easy, and requires a minimum of equipment: housing for mice, syringe, needle, and microscope. An average spread of 95% confidence limits of ±15.6% was obtained using six mice at each dosage level; ±11.1% with ten mice per level; ±9.0% with twelve mice per level; and ±8.4% with fifteen mice per level. Ten mice per dosage level were routinely used as a compromise between narrow fiducial limits and time required for each assay. This routine assay could be done in ninety minutes. The mice became less sensitive to atropine with their increasing age, weight, and number of prior injections of atropine. A straight line (log dose-response) could be obtained by utilizing suitable dilutions between those that gave a response 50% greater than maximal control readings and a response 20% less than the diameter of the pupil maximally dilated by atropine (7, 8).

In utilizing the cat eye method several observations were made that differed from the protocol listed in the Cat Eye Method—Official (5). One drop (0.05 ml.) of 1.2 mg. % atropine is stated to be the minimal effective dose. One drop of 0.75 mg. % atropine was found to be effective in this laboratory. A few (<20%) cats showed residual mydriatic effects twenty-four hours after instillation, thereby necessitating alteration of the official method from use of the cats every twenty-four hours to every forty-eight hours. It was felt that reduction of instillation volume (from 0.05 to 0.025 ml.) materially lessened the chance of accidental splashing or conjunctival overflow. This bioassay is adequate for screening of mydriatic activity, but the construction of the bioassay using small numbers of cats can give only an approximation of relative potency.

It was initially hoped that an adaptation of the mouse pupil or cat eye assay could be applied to humans. The susceptibility of blue irides and resistance of darker pigmented irides to atropine made such adaptation impossible. Hence a method comparing standard solution in one eye with an aged solution in the other eye at the same time was formulated. The human method is suitable for reasonably rapid screening of sterile, aqueous solutions of atropine provided the pH of the solution does not cause pain. For meaningful statistical conclusions at least 30 subjects would be required, thereby making the procedure somewhat impractical as a laboratory tool.

Tropine hydrochloride (pH 6) in concentrations up to 4.0 Gm. % appears to have no gross physiologic effects upon mice. A previous report states that high concentrations of intramuscular tropine caused slight mydriasis in cats (9). Solutions of 4.0 Gm. % tropine hydrochloride were administered subcutaneously, intramuscularly, intraperitoneally.

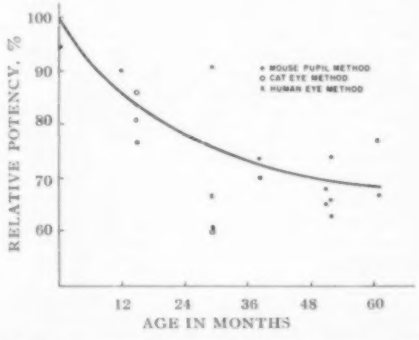

Fig. 2.—Comparison of length of aging upon relative potency.

intravenously, intrathoracically, and topically to the eye without causing mydriasis in mice. Similarly administered tropine hydrochloride did not cause inhibition or intensification of the effects of subsequently administered atropine. Mixtures of tropine base and tropic acid used in the same manner as above did not cause mydriasis or alteration of atropine effects in mice.

It is assumed that the physiologic activity observed in the bioassays is a reasonably accurate reflection of the amount of atropine in the various solutions since the hydrolytic products of atropine appear to have no mydriatic effect in mice. Hydrolysis of atropine salts at pH 5.0 ± 0.2 occurs very slowly even at 100°.

Aqueous solutions of atropine salts appear to deteriorate in physiologic activity with age. The results of the assays are plotted in Fig. 2. So few samples were available for study that the curve of deterioration cannot be drawn with confidence and any extrapolation is impossible.

No conclusions can be drawn from these data as to the relative merits of the sulfate or tartrate salts of atropine or of the various types of storage containers in resisting physiologic deterioration with age.

SUMMARY

1. Solutions of atropine salts deteriorate in physiologic activity when stored for long periods of time. Solutions five years old showed deterioration of physiologic activity of about 35 per cent.

2. Aged, aqueous solutions of atropine sulfate and tartrate were bioassayed by the mouse pupil, cat eye, and human eye methods. Results among bioassays agree. The mouse pupil method is best suited as a laboratory tool.

3. Technical considerations of three methods of atropine bioassay were discussed and compared.

4. The hydrolytic products of atropine, tropine and tropic acid, were found to have no mydriatic effects in mice. Tropine does not alter atropine effects when administered in conjunction with or just prior to injection of atropine.

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The Determination of Alkali Salts of Organic Acids by Residual Nonaqueous Titration*

By MARTIN I. BLAKE†

A residual nonaqueous titration method for the determination of alkali salts of organic acids is presented. Exactly 25 ml. of 0.1 *N* perchloric acid in glacial acetic acid is added to about 1 meq. of salt. Solution is effected and the excess perchloric acid is determined by titration with 0.1 *N* sodium acetate in glacial acetic acid. The method is applicable potentiometrically and visually. A comparison is made with the official assay procedure. Recommendations are made for the routine analysis of alkali salts of organic acids.

THE PYROLYTIC PROCEDURE for determining alkali salts of organic acids has been official (1) for over 75 years. The only major change occurred in the ninth revision of the U. S. P. when the method was altered from a direct titration to a residual determination. Numerous shortcomings of the assay have been reported in the literature. Ehman (2) pointed out that complete ignition to remove the carbon may cause appreciable amounts of the alkali carbonate to volatilize, whereas incomplete ignition may result in adsorption of carbonate by the charred matter and subsequent loss by filtration.

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Lyons (3), Jones (4), and Hitchens (5), described the procedure as a tedious and time-consuming one. Clark (6) indicated that loss due to boiling and filtration as well as the inaccuracy of the methyl orange indicator were sources of error.

Blaug (7) recently described a method for determining certain alkali salts of organic acids by means of a cation exchange resin. The column removed the alkali cation while the organic acid passed through the column and was determined by direct titration with standard sodium hydroxide solution.

The successful titration of alkali salts of organic acids by direct nonaqueous titration has been demonstrated. Fritz (8), in reviewing the subject of acid-base titrations in nonaqueous solvents, points out the limitation of determining alkali salts in an aqueous medium by titrating with a standard acid solution. However, in glacial acetic acid as a solvent and with perchloric acid as a titrant, the displacement of the organic acid in an alkali salt combination is quantitative. The routine use of sodium acetate as a basic titrant in glacial acetic acid and potassium acid phthalate as a primary standard for perchloric acid illustrates this.

Palit (9) titrated salts of monocarboxylic acids using a mixed solvent comprised of a glycol and a higher alcohol, a hydrocarbon, or a chlorinated hydrocarbon (G-H mixture). Sodium acetate, sodium salicylate, sodium benzoate, sodium mandelate, and others were studied. The titration of alkali salts of dibasic acids was not applicable because of poor solubility or because the displaced acid was sufficiently strong to effect a color change with the indicator.

Markunas and Riddick (10) analyzed 400 compounds by direct titration, potentiometrically and visually. Numerous alkali salts were investigated including acetates, citrates, salicylates, and benzoates. The poor solubility of certain salts in acetic acid was noted as a limitation of the procedure. A reproducibility of 0.2 per cent was reported.

Wollish and Pifer (11) determined a number of soluble salts in glacial acetic acid by direct titration. Sodium acetate was included in the study. A reproducibility of 0.2 per cent was indicated.

In a general paper dealing with titrations in nonaqueous solvents, Auerbach (12) successfully titrated a number of alkali salts in glacial acetic acid using perchloric acid as a titrant. Sodium citrate and potassium sodium tartrate were among those analyzed. Experimental details and data were not reported.

Beckett, Camp, and Martin (13) investigated a variety of salts in glacial acetic acid using perchloric acid as the titrant. Citrates, salicylates, tartrates, and benzoates were assayed potentiometrically and visually. A reproducibility of 0.15 per cent was noted. Only dilute solutions of tartrates could be studied because of their poor solubility in acetic acid. It was necesary to use 0.01 *N* perchloric acid as the titrant for tartrates.

The present report compares the official method for analyzing alkali salts of organic acids with a residual nonaqueous method. An excess of standard perchloric acid solution is added to the sample, solution is effected, and the excess perchloric acid determined by titration with standard sodium acetate solution in glacial acetic acid. The method is successful potentiometrically and visually. Recommendations are made for the routine analysis of these compounds.

EXPERIMENTAL

Official Assay Procedure.—Sodium tartrate ($C_4H_4Na_2O_6 \cdot 2H_2O$), analytical reagent, was selected as the alkali salt of an organic acid for this study. The salt was made anhydrous by drying at 150° for four hours and was assayed as directed in the U. S. P. (1) under "Alkali Salts of Organic Acids." The results of 10 consecutive assays are shown in Table I. Sample weights and per cent recoveries are indicated. This study served as a standard with which to compare the nonaqueous assay.

TABLE I.—THE ASSAY OF SODIUM TARTRATE BY THE OFFICIAL PROCEDURE

Sample No.	Sample Weight, Gm.	Recovery, %
1	2.0442	100.66
2	2.0319	100.26
3	2.0292	100.00
4	2.0182	99.46
5	1.8395	99.73
6	1.6140	99.43
7	1.6224	99.57
8	1.5690	99.13
9	1.5191	99.74
10	1.5431	99.88
	Average	99.79
	Std. Dev.	0.42

Residual Nonaqueous Procedure.—*0.1 N Perchloric Acid.*—Tenth-normal perchloric acid was prepared by adding 8.4 ml. of 72% perchloric acid to about 400 ml. of glacial acetic acid in a 1-liter volumetric flask. Approximately 20 ml. of acetic anhydride was added. The solution was diluted to the mark with glacial acetic acid and allowed to stand overnight. The exact normality of the perchloric acid solution was determined by standardization against reference standard potassium acid phthalate Approximately 400 mg. (about 2 meq.) of potassium acid phthalate was accurately weighed and dissolved in 50 ml. of glacial acetic acid. Heat was employed to effect solution. The mixture

was titrated potentiometrically. The average of three determinations was taken as the normality. The precision was better than 1 part per 1,000.

0.1 N Sodium Acetate.—Tenth-normal sodium acetate was prepared by dissolving 8.2 Gm. of anhydrous sodium acetate in glacial acetic acid and diluting to 1 liter with glacial acetic acid. The solution was standardized potentiometrically against the previously standardized perchloric acid solution. The average of three determinations was taken as the normality. The precision was better than 1 part per 1,000.

Assay Procedure.—Approximately 100 mg. (about 1 meq.) of anhydrous sodium tartrate was accurately weighed into a 150-ml. beaker. A stirring bar was carefully placed in the beaker. About 30 ml. of glacial acetic acid was added. This was followed by the addition of exactly 25 ml. of 0.1 *N* perchloric acid solution by pipet. The glass and sleeve type calomel electrodes of a Fisher titrimeter were placed in the solution. The beaker containing the solution was placed on a magnetic stirrer. The mixture was stirred until solution was effected (5–10 minutes). The solution, magnetically stirred, was titrated with 0.1 *N* sodium acetate solution. The volume of titrant added and millivolt readings were recorded. In the vicinity of the end point titrant was added dropwise. The titration required about 15.00 ml. of sodium acetate solution to reach the end point. The exact end point was determined by plotting $\Delta E/\Delta V$ *versus* V (ml.). The volume reading where $\Delta E/\Delta V$ was a maximum was taken as the end point. In most instances the curve obtained by plotting the volume of titrant *versus* millivolt readings permitted the detection of the end point by inspection.

Calculation of Per Cent Recovery.—The per cent recovery of sodium tartrate was readily calculated from the expression:

$$[(\text{meq } HClO_4 - \text{meq } NaC_2H_3O_2)\,97.03/\text{sample weight}] \times 100 = \%\text{ recovery} \quad (1)$$

where

meq $HClO_4$ = 25 ml. × normality of the perchloric acid solution

meq. $NaC_2H_3O_2$ = volume (ml.) of sodium acetate solution consumed in back titration × normality of the sodium acetate solution

97.03 = milliequivalent (meq.) weight of anhydrous sodium tartrate

sample weight = weight of sample in mg.

Sample weights and per cent recoveries for ten consecutive assays are shown in Table II.

Determination of Other Alkali Salts of Organic Acids.—The nonaqueous procedure described in the previous section was applied to other alkali salts of organic acids. The salts studied were commercial grade and were not further purified. The anhydrous alkali salts (1–2 meq.) were assayed as described under sodium tartrate. The results of the assays are shown in Table III. Milliequivalent weights and per cent recoveries are listed. The per cent recoveries were calculated by substituting the data in Eq. 1, using the proper milliequivalent weight shown in Table III, and are average values of at least 3 determinations.

TABLE II.—THE ASSAY OF SODIUM TARTRATE BY RESIDUAL NONAQUEOUS TITRATION

Sample No.	Sample Weight, mg.	Recovery, %
1	109.4	100.46
2	102.5	99.51
3	108.9	99.44
4	124.0	99.44
5	104.5	99.81
6	91.9	100.54
7	163.5	100.11
8	118.7	100.24
9	108.6	100.00
10	133.7	99.63
	Average	99.92
	Std. Dev.	0.39

TABLE III.—THE ASSAY OF ALKALI SALTS OF ORGANIC ACIDS BY RESIDUAL NONAQUEOUS TITRATION

Alkali Salt	Milliequivalent Weight (Anhydrous Salt), mg.	Average Recovery, %	Average Deviation, %
Potassium sodium tartrate	105.09	98.59	0.33
Potassium acid tartrate	188.18	97.76	0.14
Potassium acetate	98.15	97.32	0.31
Potassium citrate	102.14	97.64	0.16
Lithium citrate	69.97	99.33	0.28
Sodium benzoate	144.11	96.87	0.16
Sodium citrate	86.03	101.50	0.25
Lithium benzoate	128.05	97.00	0.27
Sodium salicylate	160.11	97.40	0.15

Indicator Method.—*Indicator Solution.*—Methyl violet indicator solution was prepared by dissolving 1.0 Gm. of methyl violet crystals in 100 ml. of glacial acetic acid.

Sodium tartrate and the salts listed in Table III were assayed potentiometrically in the manner previously described, but, in addition, two drops of indicator solution were added at the start of the titration. The color change of the solution at the graphic end point was noted. Another series of titrations was conducted without the use of the titrimeter. The end point was determined on the basis of color change alone. For each of the salts studied the color change at the end point was from a blue-green to a blue color or the first permanent blue showing no trace of green. One or two drops of titrant were sufficient to effect this color change at the end point. The end point of the visual titration agreed within 0.03 ml. of the potentiometric end point which was determined graphically. The concentration of the indicator solution was an important factor. Solutions of methyl violet ranging in concentration from 0.1 to 2.0% in glacial acetic acid were tested. Two or three drops of a 1.0% solution gave the most satisfactory color change at the end point.

DISCUSSION

The official assay for alkali salts of organic acids is tedious and time-consuming. The ignition, boiling, and filtration procedures are possible sources of error. Each manipulation requires careful attention. Despite these factors, the results (Table I) show good precision and are in excellent agreement with the results (Table II) of the nonaqueous method. An average per cent recovery of 99.79 ± 0.42 was obtained for the official assay. For routine analysis, four assays, run concurrently, require about three and a half hours.

The direct titration of alkali salts in nonaqueous media has been reported by numerous workers. Several advantages are apparent. Considerably less time is required for the analysis. There are fewer and less tedious manipulations involved and, thus, less chance for indeterminate errors. Smaller sample weights are needed. The official procedure calls for a 2-Gm. sample. Less than 100 mg. can be accurately determined by the nonaqueous method. Accuracy and precision are comparable. A limitation, however, is the poor solubility of certain salts. The tartrates, for example, are poorly soluble in glacial acetic acid. Although the visual titration is simpler to carry out than the potentiometric method, there is some difficulty in observing a suitable end point when methyl violet is used as the indicator. The change from a blue to a blue-green color is not readily detectable.

The residual nonaqueous method described in this paper yielded excellent results for sodium tartrate (Table II). Solubility was no problem. Complete solution was effected in 5–10 minutes without the use of heat. The average per cent recovery was 99.92 ± 0.39. The method is equally applicable to other tartrates and other alkali salts of organic acids as shown in Table III. When an indicator was used to detect the end point results comparable to the potentiometric procedure were obtained. One or two drops of titrant were sufficient to cause a change in color from blue-green to blue at the end point. The end point appears to be more discernible by the residual method than by the direct titration. The potassium salts caused the solution to become cloudy due to formation of insoluble potassium perchlorate. This did not interfere with the observation of the end point.

For the routine analysis of alkali salts of organic acids two procedures are recommended. The first is a potentiometric method using a Fisher titrimeter or similar instrument. A preliminary titration is conducted on the salt being analyzed. The voltage which corresponds to the end point is noted. For routine determinations the voltage scale knob is set at the end point value as determined in the preliminary run. Titrant is added to the solution until the magic eye of the titrimeter just opens. The volume of titrant is substituted in Eq. 1. The per cent recovery is determined using the proper meq. value.

In the second procedure 2–3 drops of indicator solution is added to the solution in place of using the titrimeter. Titrant is added until the first permanent blue color appears. The volume of titrant added is substituted in Eq. 1 as described in the previous procedure. Four assays can be conveniently carried out in less than one hour by either procedure.

SUMMARY

1. The official assay for alkali salts of organic acids is compared with a residual nonaqueous method. Sodium tartrate served as the alkali salt for the comparison.

2. The nonaqueous method is applied to a variety of alkali salts. The procedure is particularly suitable for tartrates. Tartrates are poorly soluble in glacial acetic acid and are not readily determined by direct titration. The method is applicable both potentiometrically and visually.

3. General procedures are presented for the routine analysis of alkali salts of organic acids.

4. The indicator method, as described in this paper is an experiment now incorporated in our course in drug assay. It is run in conjunction with the official procedure for the assay of alkali salts of organic acids. The results have been favorable.

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A Comparative Study of the Local Anesthetic Potencies and Toxicities of *Beta*-Diethylaminoethyl Cumate and Procaine*

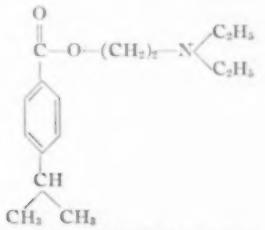
By GUILFORD C. GROSS† and ELBERT VOSS‡

Beta-diethylaminoethyl cumate was found to be somewhat more toxic than procaine as a local anesthetic. Its acute toxicity in mice was less than that of procaine. It was also more potent than procaine on the basis of effective concentrations as determined in human intradermal tests. Given by subarachnoid injection in rabbits, little, if any, difference in spinal anesthetic potency was observed. The LD by intraperitoneal injection in mice was determined. Procaine hydrochloride was definitely less irritating than the cumate ester as determined by human intradermal tests, application to the rabbit eye, and by the trypan blue skin irritation test of Hoppe, *et al.*

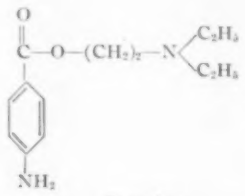
BETA-DIETHYLAMINOETHYL CUMATE was found to be somewhat more potent than procaine as a local anesthetic when these compounds were compared by intradermal tests on human subjects. Its acute toxicity by intraperitoneal injection in mice was less than that of procaine but, on the other hand, it was found to be definitely more irritating to tissues.

β-Diethylaminoethyl cumate is a synthetic compound possessing the general chemical configuration that is characteristic of many local anesthetic agents, i. e., tertiary amino esters of aromatic acids. It differs from procaine only in that its acid moiety is cumic acid rather than para-aminobenzoic acid.

Williams and Voss (1) in 1951, following a study of the local anesthetic properties and toxicities of several esters of cumic acid, suggested that β-diethylaminoethyl cumate held promise of becoming a useful addition to the field of local anesthetics. They found that this compound, on the basis of local anesthetic potency, acute toxicity, and irritation, compared favorably with procaine. The other esters studied, β-dimethylaminoethyl cumate and γ-diethylaminopropyl cumate, while possessing local anesthetic activity, were considered by them to be inferior to the β-diethylaminoethyl compound. The present study of β-diethylaminoethyl cumate, prompted by this favorable report, was undertaken to supplement their work in an attempt to better evaluate the compound in relation to known local anesthetics. To this end, further studies have been conducted on local anesthesia, toxicity, and irritation, procaine being used as the standard for comparison in these texts.



β-Diethylaminoethyl cumate



Procaine

EXPERIMENTAL PROCEDURES

Preparation of β-Diethylaminoethyl Cumate.—The β-diethylaminoethyl cumate used in these studies was prepared in the form of the hydrochloride according to the method of Bryan and Foote (2). Purification of the salt was accomplished by recrystallization from ethanol.

Local Anesthesia: Human Intradermal Tests.—The object of these tests was twofold: (*a*) to determine the minimal effective concentrations of β-diethylaminoethyl cumate and procaine and (*b*) to determine the duration of anesthesia produced by various concentrations of each. Six male and two female volunteers were used in the tests. Each sub-

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ject, with but one exception, received injections of both drugs in randomly assigned concentrations. Injections were made on the flexor surfaces of the forearms after cleansing the area with 70% alcohol. Solutions were prepared in $M/8$ sodium chloride and the volume of each injection maintained at 0.1 cc. The usual procedure was to make 3 injections of a given concentration of the drug across the arm, test these for anesthesia, then make another series of 3 injections using a different concentration and so on for a total, in most instances, of 9 injections representing 3 concentrations of drug. In testing for anesthesia, two types of stimuli were employed: (*a*) a sewing needle to test the response to pain, following the method of McIntyre and Sievers (3) and (*b*) a wisp of cotton, twisted to a fine point, to test the response to touch according to the method of Sollmann (4). All tests were made at two-minute intervals until the absence of anesthesia was definitely established.

Spinal Anesthesia in Rabbits.—Subarachnoid injections of β-diethylaminoethyl cumate HCl and procaine HCl were made in rabbits according to the technique of Bieter, *et al.* (5). For this study, young rabbits, 0.8 to 1.5 Kg., random sex, were used. These animals were found to be somewhat more convenient to use than the larger, mature rabbits. Solutions of the drugs were prepared in distilled water, 0.02 cc. of solution being injected per centimeter of spinal length. Animals were observed for the presence of motor paralysis. This was regarded as being complete when the muscles of the hind legs were completely flaccid, the animals being unable to use or move the hind legs. Incomplete motor paralysis was recorded when the animals had some use of these muscles, but dragged the hind legs perceptibly in moving. For the purpose of determining the duration of motor paralysis, the end point was taken as that time when the animal was again able to hop about. A number of animals had to be discarded because of doubt that a successful injection had been made. Excessive struggling by the animal, or the withdrawal of blood following an injection was cause for rejection.

Acute Toxicity.—Median lethal doses of β-diethylaminoethyl cumate HCl and procaine HCl were determined by intraperitoneal injections in male, Swiss strain mice, 16 to 23 grams in weight. Determinations were made in an air-conditioned room, maintained at 22°. The method of Miller and Tainter (6) was used for the estimation of the LD_{50}.

Irritation Tests on the Rabbit Eye.—Sixteen rabbits were used in testing four concentrations each of β-diethylaminoethyl cumate HCl and procaine HCl. Using two rabbits for each level of drug, 0.1 ml. of the drug solution was instilled in the conjunctival sac of the left eye, the right eye remaining as the control. After instillation of the solution, the lid was held closed for a period of one minute before the animal was released. Eyes were examined for signs of irritation at the end of one, twenty-four, and forty-eight hours.

Trypan Blue Test for Irritation.—This test, described by Hoppe, Alexander, and Miller (7), was employed for comparing the irritant properties of β-diethylaminoethyl cumate HCl and procaine HCl. Ten albino rabbits of random sex and weighing 0.8 to 2.0 Kg. were used in testing three concentrations of each of the two compounds. Each animal received a total of six injections, one for each concentration of each drug. Drug solutions were prepared in normal saline. The method of Luduena and Hoppe (8) was used in scoring the tests and the Threshold Irritant Concentration 4 (TIC) was determined for each compound.

RESULTS AND DISCUSSION

Human Intradermal Tests.—The results of 72 intradermal injections of β-diethylaminoethyl cumate HCl and 60 of procaine HCl are summarized in Table I. It is apparent from these data that the response to pain was more precise than the response to touch. In some instances, it was found that wheals would show anesthesia to one stimulus but not to the other, a discrepancy which was occasionally observed even with the higher concentrations of drug. Thus, two wheals of 1/2% β-diethylaminoethyl cumate HCl (in two different individuals) showed anesthesia to pain of 12 and 16 minutes duration, respectively, yet no anesthesia was reported to touch. A possible explanation is that hair at the site of injection interfered with the touch test; a disturbance of the hair by the wisp of cotton

TABLE I.—HUMAN INTRADERMAL TESTS

Concn., %	*Beta*-Diethylaminoethyl Cumate HCl: No. Showing Anesthesia	Duration of Effect: Mean, min.	Range, min.	Coefficient Variation	Procaine HCl: No. Showing Anesthesia	Duration of Effect: Mean, min.	Range, min.	Coefficient Variation
Response to Pain								
1/2	12/12	12.2 ± 0.72	8–16	20.4	12/12	11.5 ± 0.74	6–16	22.3
1/4	12/12	10.0 ± 0.55	8–14	19.1	12/12	8.2 ± 0.38	6–10	16.3
1/8	12/12	7.7 ± 0.59	4–12	26.9	12/12	5.8 ± 0.46	4– 8	27.3
1/16	12/12	5.7 ± 0.42	4– 8	25.4	8/12	1.5 ± 0.36	0– 4	82.7
1/32	11/12	3.5 ± 0.56	0– 6	55.1	2/12	0.33 ± 0.22	0– 2	233.3
1/64	6/12	1.0 ± 0.30	0– 2	104.0	...	...	...	...
Response to Touch								
1/2	10/12	9.0 ± 1.58	0–16	61.0	12/12	11.0 ± 0.94	4–16	29.5
1/4	12/12	10.3 ± 0.59	8–14	19.9	12/12	9.2 ± 1.29	4–18	48.7
1/8	11/12	7.3 ± 0.90	0–10	42.4	11/12	5.8 ± 0.72	0– 8	42.5
1/16	11/12	4.7 ± 0.67	0– 8	49.5	8/12	3.0 ± 0.76	8– 6	87.7
1/32	10/12	3.5 ± 0.70	0– 9	69.4	5/12	1.8 ± 0.75	0– 8	143.2
1/64	6/12	1.7 ± 0.59	0– 6	123.4	...	...	...	...

TABLE II.—SPINAL ANESTHESIA IN RABBITS

Concn., %	*Beta*-Diethylaminoethyl Cumate HCl			Procaine HCl		
	No. Showing Motor Paralysis	Approx. Duration Paralysis, min.		No. Showing Motor Paralysis	Approx. Duration Paralysis, min.	
		Mean	Range		Mean	Range
2	2/2	35.0	20–50	4/4	27.5	15–35
1	3/3	20.0	15–25	4/5	15.0	0–20
1/2	4/4	12.5	10–15	2/3	8.3	0–15
1/4	2/8	2.0	0–10	3/4	5.0	0–10
1/8	2/2	5.0	5	3/3	8.3	5–10
1/16	2/3	3.3	0–5	1/2	2.5	0– 5

being felt by the individual while the actual touch of the cotton to the skin was not. He may thus have reported "negative anesthesia" when actually anesthesia was present. Although care was taken to avoid this source of error, it may not have been entirely eliminated.

A number of "doubtful" responses were encountered in the tests. These usually occurred with the lower concentrations of drug or, in the case of the higher concentrations, during the terminal periods of anesthesia. In most instances, such tests were followed by a definitely negative reaction. In tabulating the data in Table I, doubtful responses were uniformly considered positive on the assumption that some anesthesia existed although it was incomplete.

Erythema was a commonly observed sign of irritation following injections of β-diethylaminoethyl cumate. All subjects who received 1/4% and 1/2% concentrations of the drug showed this reaction, and in some instances the areas involved were quite extensive. Two of four subjects exhibited slight erythema around the 1/64% wheals. In one individual, definite swelling was noted around the sites of the 1/8, 1/4, and 1/2% injections. Another person reported itching around 1/4% wheals. In most instances, the wheals disappeared rapidly and healing was uneventful. Neither induration nor eschar formation was observed in any case. Procaine appeared to be definitely the less irritating of the two compounds. Two individuals who received procaine exhibited erythema, one from concentrations as low as 1/32%. These, however, were the only signs of irritation noted from procaine.

The relative potencies of the two drugs are estimated from the regression lines shown in Fig. 1. Defining the "minimal effective concentration" as that strength which would produce anesthesia in 50% of the wheals, the value for β-diethylaminoethyl cumate HCl is estimated to be 0.016% and that for procaine HCl, 0.046%. Although it is felt that these values serve to show the approximate relationship between the anesthetic strengths of the two drugs, it is apparent that this expression of relative potencies cannot be regarded as being more than an approximation.

Spinal Anesthesia in Rabbits.—The results of 43 spinal injections, recorded in Table II, indicate little difference in the activities of the two compounds. Both usually produced complete motor paralysis in concentrations of 0.5% and above, and incomplete or no paralysis in concentrations less than 0.5%. Also, for a given concentration of drug, there appeared to be little, if any, difference in the duration of motor paralysis.

Acute Toxicity.—The LD_{50} for β-diethylaminoethyl cumate HCl was found to be 470 ± 13.4 mg./Kg. and that for procaine HCl 189 ± 10.0 mg./Kg. The corresponding values obtained by Williams and Voss (1) for these compounds are 370 ± 18.2 mg./Kg. and 275 ± 12.6 mg./Kg. While these results are in poor agreement, both show β-diethylaminoethyl cumate to be less toxic than procaine.

The symptoms of acute toxicity were essentially the same for both compounds. With adequate doses, convulsions occurred which were both clonic and tonic in character. A period of lethargy, with marked respiratory depression would follow the convulsion. Usually, an animal that survived this period would recover completely. Procaine treated mice seemed to recover more rapidly than did those receiving β-diethylaminoethyl cumate.

Irritation Tests on the Rabbit Eye.—The results for β-diethylaminoethyl cumate are shown in Table III. Procaine HCl which was tested in the same concentrations as β-diethylaminoethyl cumate HCl, i. e., 10, 5, 2, and 1%, showed no evidence of being irritating. Since β-diethylaminoethyl cumate HCl was definitely irritating in concentrations of 2% and above, it compares rather poorly with procaine in this respect.

TABLE III.—EYE IRRITATION TESTS ON RABBITS, *Beta*-DIETHYLAMINOETHYL CUMATE HCl

Concn., %	1-hr. Observation			24-hr. Observation			48-hr. Observation		
	Redness	Chemosis	Disch.	Redness	Chemosis	Disch.	Redness	Chemosis	Disch.
10	xx	xxx	x	xx	xx	xxx	x	0	0
10	xx	xxx	xx	xxx	xxx	xxx	x	0	x
5	x	xx	xx	x	x	0	0	0	0
5	xx	xx	x	x	x	0	0	0	0
2	x	x	x	0	0	0	0	0	0
2	x	x	x	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0

x = slight, xx = moderate, xxx = marked.

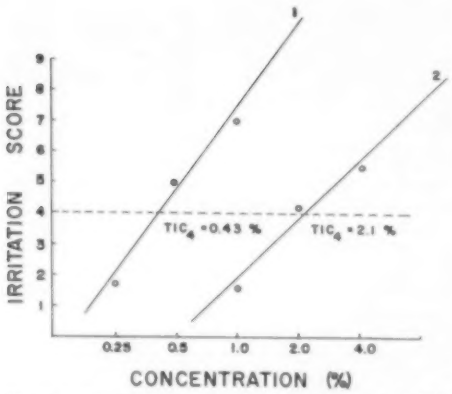

Fig. 1.—Trypan blue test for irritation. 1—β-Diethylaminoethyl cumate HCl. 2—Procaine HCl.

Trypan Blue Test for Irritation.—From Fig. 1, the TIC_4 for β-diethylaminoethyl cumate HCl was found to be 0.43% as compared with 2.1% for procaine. Thus, again, β-diethylaminoethyl cumate gave evidence of being considerably more irritating.

SUMMARY

1. The local anesthetic potencies, acute toxicities, and irritant properties of β-diethylaminoethyl cumate HCl and procaine HCl have been compared.

2. β-Diethylaminoethyl cumate HCl appeared to be the more potent of the two compounds as a local anesthetic when compared on the basis of their minimal effective concentrations as determined in human intradermal tests. Little, if any, difference in potency was observed in their abilities to produce motor paralysis in rabbits when given by subarachnoid injection.

3. β-Diethylaminoethyl cumate HCl was found to have a lower acute toxicity to mice than procaine HCl. Their respective LD_{50}'s by intraperitoneal injection are 470 ± 13.4 mg./Kg. and 189 ± 10.0 mg./Kg.

4. Irritation tests showed procaine HCl to be definitely the less irritant of the two compounds. In intradermal injections in humans, β-diethylaminoethyl cumate HCl in concentrations less than $^1/_2$ per cent commonly produced erythema while procaine HCl rarely gave this reaction. In tests on the rabbit eye, 2 per cent solutions of the former compound were found to be definitely irritating. Procaine HCl, on the other hand, gave no evidence of irritation in concentrations up to 10 per cent. The trypan blue test confirmed the fact that β-diethylaminoethyl cumate HCl was actually considerably more irritating than procaine HCl since it required approximately five times the concentration of the latter drug to produce a degree of irritation corresponding to that for the cumic acid ester.

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Gitoside—A New Digitalis Glycoside*

By JAMES E. MURPHY

A new glycoside, the monodigitoxoside of gitoxigenin, has been isolated from *D. lanata*. Procedures for its isolation and characterization are given.

FROM RESULTS of paper chromatographic analyses of extracts of digitalis run in our laboratories as well as those published by others, it became evident that besides the known glycosides of digitalis there are many other glycosidal substances present which have not been isolated and characterized. During the past few years, several new glycosides, gitorin (1), strospeside (2, 3), digipurpurin (4), digifolein (4), glucogitofucoside (4), gitaloxin (5), verodoxin (6), digiproside (7), and diginatin (8), have been isolated. In this paper we shall describe another glycoside which has not been previously isolated and which we have obtained from extracts of *D. lanata*.

* Received August 3, 1956, from the Analytical Research Laboratory, Burroughs Wellcome & Co. (U.S.A.) Inc., Tuckahoe, N. Y.
The author wishes to express his gratitude to Dr. A. Elek for the microanalyses, to Miss W. Holden for the bioassay, and to Dr. C. W. Ferry for his advice and encouragement during this work.

This new glycoside has also been detected in extracts of *D. purpurea*, *D. thapsi*, *D. sibirica*, *D. lutea*, *D. ambigua*, *D. mertonensis*, and *D. ferruginea*. It is also one of the substances present in commercial amorphous gitalin. It has been given the name gitoside and was originally called Unknown "B" in an earlier paper from this laboratory. Its position on paper chromatograms was indicated in Fig. 1 of that publication (8).

The position of gitoside on paper chromatograms corresponds very well with that of the unknown glycoside (*b*) detected by Jensen (9), and it may be the same substance. He reported that it gave a fluorescent reaction when sprayed with trichloroacetic acid in alcohol, like the gitoxigenin-containing glycosides, and a green color given by glycosides containing 2-desoxy sugars, such as digitoxose. In a later publication (10), Jensen redesignated this as glycoside (B_4), isolated it in microgram amounts by paper chromatography and showed that on heating it with dilute sulfuric acid, gitoxigenin was formed.

Gitoside was isolated in our laboratory by means of partition chromatography on Celite® columns from residues obtained in the processing of digoxin. The *D. lanata* from which they were obtained was first year growth, oven dried leaf. Analytical data before and after hydrolysis showed that the glycoside consisted of a mole of gitoxigenin and a mole of digitoxose. Other monosides of gitoxigenin have been found in digitalis, e. g., strospeside is the monodigitaloside of gitoxigenin and gitorin is the monoglucoside of gitoxigenin.

At first, results indicated that the substance could be the gitalin described by Kraft and Cloetta (11, 12), because assays indicated a ratio of approximately 2:1 digitoxose-gitoxigenin. It was found, however, that the sample was not purified sufficiently and the presence of digoxin increased the digitoxose result considerably. From results of our work on both *D. lanata* and *D. purpurea*, there does not appear to be a glycoside of the composition of gitalin in digitalis unless it is formed as an artifact because of the methods used for the extraction and isolation.

Using a modification of the quantitative assay procedure worked out by Jensen for gitoxin (13), the amount of gitoside in a sample of dried *D. lanata* leaves was found to be 0.015 per cent.

Bioassay showed it to be cardioactive; a U. S. P. XV pigeon assay giving a lethal dose of 1.12 mg./Kg.

EXPERIMENTAL

First attempts at isolation of the unknown substance, which had been discovered by paper chromatography of some residues from digoxin processing, were made by treating the residues with acetone, in which it appeared quite soluble. This freed it from most of the digoxin and a concentrate of this solution was put on a partition column consisting of Celite containing water and formamide as the stationary phase and developed with chloroform-benzene 3:1. The fractions containing the unknown glycoside were worked up and yielded a white compound, m. p. 212°. Paper chromatographic analyses indicated that it was a single substance and other data suggested that it was composed of two moles of digitoxose and 1 mole of gitoxigenin. However, after hydrolysis, considerable digoxigenin showed on chromatograms of the hydrolysate indicating that the separation from digoxin had not been complete. After development of a more sensitive spray reagent for digoxin,[1] this was definitely proved to be the case. Attempts at separating digoxin from the unknown by rechromatographing with the same or similar solvent systems was not successful.

It was decided to use paper chromatography as a screening method for testing other solvent systems. Formamide saturated strips were used. Mixtures of methylene chloride and acetone or ethylene chloride and acetone showed promise and instead of having a lower R_f value than digoxin as was the case with chloroform and chloroform and benzene, the unknown had a higher R_f value. The R_f values were quite high and it was found that by adding benzene the development was slowed to a rate which would warrant using a mixture of this composition for a column separation.

Using a system of ethylene chloride 10 vol., benzene 10 vol., acetone 2 vol., and a ratio of formamide-water 2:1 for the stationary phase, digoxin could be completely separated from the unknown. Partitioning the residue on this column yielded the unknown contaminated with small amounts of gitoxin and gitoxigenin. These were removed by rechromatographing on a column using the same stationary phase but a mobile phase of chloroform containing 10% *n*-heptane. The proper fractions were concentrated, the concentrate poured into cold water, filtered off, and dried. The dry precipitate was refluxed with a small amount of acetone to remove much of the color and then dissolved in hot 80% alcohol, treated with charcoal, and allowed to crystallize slowly. Filtration and drying yielded a white, crystalline powder, individual particles appearing as light yellow rosettes under the microscope, m. p. 215–217°.

Anal.—Calcd. for $C_{29}H_{44}O_8$: C, 66.90; H, 8.52. Found C, 66.60; H, 8.73. Specific rotations were determined in a 2 dm. tube: $[\alpha]_D^{26}$ +7.8°, $[\alpha]_{5461}^{26}$ +10.4° (C = 4.1, methyl alcohol), $[\alpha]_D^{26}$ −18.9°, $[\alpha]_{5461}^{25}$ −21.8° (C = 5.9 pyridine).

It gave a positive reaction for a 2-desoxy sugar with Keller-Kiliani reagent. The ring test for digitalis glycosides gave a bright red color at the junction of the two layers of acid as well as in the sulfuric acid layer and the acetic acid layer changed to indigo on standing.

When assayed against digoxin using a modified Keller-Kiliani reagent, it assayed 25.0% digitoxose.

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[1] Twenty per cent trichloroacetic acid in chloroform to which 4 drops of 30% hydrogen peroxide was added just before use.

Assayed against gitoxigenin by a fluorescence assay (14), it assayed 75.9% gitoxigenin. Theory for gitoxigenin and 1 mole of digitoxose is digitoxose 25% and gitoxigenin 75%. The ultraviolet spectrum was determined in 50% alcohol and is shown in Fig. 1.

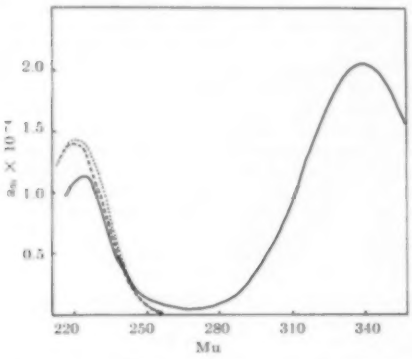

Fig. 1.—Ultraviolet Spectra. ..... Gitoside in 50% alcohol. - - - - Gitoxigenin from gitoside in alcohol. —Dianhydro gitoxigenin from gitoside in alcohol.

Hydrolysis of Gitoside.—One-half gram was refluxed with 25 ml. of alcohol, 25 ml. of water, and 0.2 ml. of hydrochloric acid for thirty minutes. The mixture was then cooled, neutralized with sodium hydroxide and concentrated under reduced pressure to about 10 ml. A large amount of aglycone separated.

The mixture was filtered and the dried precipitate amounted to 0.32 Gm. The filtrate was extracted with two 10-ml. portions of chloroform. The chloroform was evaporated and the residue was dissolved in acetone, diluted with water, heated to remove the acetone, cooled, and filtered. This gave 0.04 Gm. more aglycone for a combined yield of 97.3% of theory. The aglycone was recrystallized from methanol, m. p. 227–228°. A mixed melting point with a known sample of gitoxigenin showed no depression. Paper chromatography also suggested it was gitoxigenin. The ultraviolet spectrum is given in Fig. 1.

Anal.—Calculated for $C_{23}H_{34}O_5$: C, 70.74; H, 8.78. Found: C, 70.51; H, 8.70.

For further identification the dianhydro derivative was formed. Fifty milligrams of the aglycone was stirred with 10 ml. of propylene glycol-hydrochloric acid 1:1 until dissolved and kept at 15–20° for one hour. Forty milliliters of water was added; the mixture was cooled and filtered. The precipitate was recrystallized from 50% alcohol. Yield, 28.5 mg., m. p. 215°. A mixed melting point with a known sample of dianhydrogitoxigenin showed no depression. $[\alpha]_D^{24}$ +589° (C = 0.26, methyl alcohol). Reported for dianhydrogitoxigenin, +573° (methyl alcohol). The ultraviolet spectrum is given in Fig. 1.

The chloroform extracted filtrate from the above hydrolysis was evaporated to dryness under reduced pressure, the residue was extracted with 10 ml. of pyridine, and the pyridine was removed under reduced pressure.

Paper chromatography of the residue, using a solvent system of *n*-butyl alcohol-acetic acid-water 4:1:5, gave two spots for 2-desoxy sugars when sprayed with trichloroacetic acid in alcohol. One at R_f 0.59 corresponded to digitoxose. The other was at R_f 0.83. It was found that when the hydrolysis and paper chromatography were repeated on the aqueous portion of the hydrolysate, the ratio of the intensity of the two spots differed. This suggested that the extra spot was caused by degradation of part of the digitoxose or by formation of an α or β pyranose form of digitoxose. To check this, a sample of pure digitoxose, giving only one spot on paper chromatograms was subjected to the hydrolysis procedure. The same two spots were obtained after the treatment. Similar results were obtained when digoxin was hydrolyzed by the same procedure. Isolation of this second hydrolysis product is being attempted and it is hoped to report on it at a later date.

The benzimidazole derivative of the residue from the hydrolysis was made using the procedure of Dimler and Link (15), m. p. 210–210.5°. A mixed melting point with a known sample of the benzimidazole of digitoxose showed no depression.

SUMMARY

1. A new cardioactive glycoside which has been named gitoside has been isolated from *D. lanata*. It has been detected in several other species of digitalis including *D. purpurea*.

2. Analyses and hydrolysis of this glycoside show it to consist of 1 mole of gitoxigenin and 1 mole of digitoxose.

3. On hydrolyzing glycosides containing digitoxose part of the digitoxose forms another compound which still gives the reaction for a 2-desoxy sugar on paper chromatograms.

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Analysis of Atropine and Its Hydrolytic Products by Filter Paper Chromatography*

By HERBERT SCHRIFTMAN and ALBERT A. KONDRITZER

Paper partition chromatographic methods for the microanalysis of atropine in blood and other biological tissues and fluids are presented. In one method a *n*-butanol-HCl-H_2O (5:1:3) solvent system is employed and a modified Dragendorff reagent locates atropine as well as its hydrolytic product, tropine, as red spots on a light orange background. In the other method, the detection and analysis of micro amounts of atropine, tropine, and tropic acid are accomplished simultaneously by using a *n*-butanol-acetic acid-H_2O (10:1:6) solvent system and a spray reagent containing bromphenol blue. A photoelectric densitometer is used for the quantitative analysis of the completed chromatograms.

ATROPINE and its hydrolytic product, tropine, were separated from mixtures of alkaloids by Munier (1) by the technique of paper partition chromatography. After development of the chromatogram with a water-saturated *n*-butanol-HCl solvent the alkaloids were detected by spraying the paper (Durieux No. 122 equivalent to Whatman No. 1) with a modified Dragendorff reagent, i. e., a mixture of bismuth subnitrate and potassium iodide in acetic acid. The alkaloids formed red spots on a pale orange background. Brindle, *et al.* (2), using buffered filter paper for the separation of the alkaloids and Munier's method for the development of the chromatogram, were able to detect five micrograms of atropine. Brindle suggested that instead of spraying, a better chromatogram could be obtained by running the paper through the reagent in a manner similar to that employed in developing photographic film. Gore and Adshead (3), in their studies on the separation of alkaloids by means of paper partition chromatography, used as a developing solvent the upper layer of a mixture of 40 volumes of *n*-butanol, 50 volumes of water, and 10 volumes of glacial acetic acid; the lower aqueous layer was placed separately in the bottom of the chromatographic cabinet in order to obtain an equilibrated atmosphere. They also substituted formic and propionic acid for acetic acid in the developing solution, and used iodine solution as their spray reagent. Mannering and co-workers (4) applied paper chromatography to the detection of opium alkaloids in urine and tissues.

The present study has resulted in a micro method for the quantitative determination of atropine and tropine by measuring with a photoelectric densitometer the intensity of the color of the spot developed on the filter paper by established chromatographic procedures. We have also determined atropine, tropine, and tropic acid by spraying the chromatogram with a reagent containing a conventional acid-base indicator.

* Received August 10, 1956, from the Physiology Division, Chemical Warfare Laboratories, Army Chemical Center, Maryland.

EXPERIMENTAL

Determination of Atropine and Tropine

Individual solutions containing 0.1 to 10 micrograms of atropine alkaloid U. S. P., atropine sulfate, U. S. P., and tropine (obtained by hydrolyzing atropine alkaloid, U. S. P. with 0.5 *N* NaOH) were applied to Whatman No. 1 chromatographic paper, $18^1/_4 \times 22^1/_2$ in. A *n*-butanol-HCl-H_2O solvent system was prepared by shaking 100 cc. of *n*-butanol and 20 cc. of concentrated HCl with 60 cc. of water in a separatory funnel. At the time the paper was positioned in the chromatographic chamber, the separated water and alcohol layers were placed in beakers on the bottom of the cabinet and allowed to stand for two hours. This procedure insured equilibrium between the vapor and liquid phases, thus avoiding distortion of the chromatogram. The alcohol layer was then poured into the solvent trough and allowed to descend along the machine direction of the paper for 20 hours. The developed chromatogram was air-dried for two hours and then sprayed with a modified Dragendorff reagent prepared from two solutions: solution 1 contained 0.85 Gm. of bismuth subnitrate dissolved in 40 ml. of water and 10 ml. of glacial acetic acid; solution 2 contained 8 Gm. of KI in 20 ml. of water. Just before use, 5 ml. each of solutions 1 and 2 were mixed with 20 ml. of glacial acetic acid and 100 ml. of water. An air-controlled spray flask was used to apply the reagent to the paper.

Qualitative Results.—Atropine base and atropine sulfate formed red spots on a pale orange background, whereas the tropine spots were of a characteristic cherry-red color. One microgram was the minimum detectable amount of alkaloid in most instances. The average R_f value for both atropine and atropine sulfate was 0.95; for tropine the R_f value was 0.70. These figures agree with those reported by Munier and others (1–3). Owing to slight deviations from these R_f values caused by variations in temperature and in the filter paper, the R_f values of spots developed from experimental solutions were compared on the same sheet of

TABLE I.—DETERMINATION OF ABSORPTION MAXIMA FOR ATROPINE AND TROPINE SPOTS BY MEASURING THEIR MAXIMUM COLOR DENSITY READINGS ON A PHOTOVOLT DENSITOMETER

	Wavelength of Filter, mμ	Maximum Density Reading
Tropine (25 μg.)	530	0.00
	550	0.03
	570[a]	0.08
	600	0.04
	630	0.02
Atropine (25 μg.)	420	0.04
	440	0.06
	465	0.07
	495	0.08
	515	0.11
	530[a]	0.12
	550	0.09
	570	0.05
	600	0.03
	630	0.02

[a] Region of maximum absorption.

paper with those developed from standard solutions of the alkaloids.

Quantitative Results.—Block (5) lists several acceptable methods for scanning developed papers and quantitatively analyzing the spots. In our study a Photovolt densitometer Model 525 was used that permits readings on very small spots, by the use of narrow band filters transmitting light in the visible range, and semiautomatically plots complete density curves. Table I gives the absorption values obtained for the atropine and tropine spots when the color filters provided with the instrument are used. From these data, the color filters transmitting light in the region of maximum absorption were selected for the atropine and tropine spots, specifically a 530 mμ filter for atropine and a 570 mμ filter for tropine. Spots were developed on the same chromatogram from individual solutions containing known amounts of atropine and tropine (2 to 25 μg.). The total color density of each spot, which was measured as area under the density curve in square millimeters, was directly proportional to the amount of alkaloid in the solutions chromatographed, as shown in Table II.

TABLE II.—RELATIONSHIP BETWEEN CONCENTRATION AND TOTAL COLOR DENSITIES OF ATROPINE AND TROPINE SPOTS OBTAINED BY PAPER PARTITION CHROMATOGRAPHY

	Concn., μg.	Total Color Density (area under curve in mm.²)	Density/ Concn. = K
Atropine (using a 530 mμ color filter)	2	6	3.00
	5	16	3.20
	10	31	3.10
	15	49	3.27
	25	75	3.00
		Average	3.11
Tropine (using a 570 mμ color filter)	2	3	1.50
	5	7	1.40
	10	15	1.50
	15	23	1.53
	25	35	1.40
		Average	1.46

Determination of Atropine, Tropine, and Tropic Acid

Several methods whereby acid-base indicators are used to detect aromatic acids on chromatograms have been listed (5). Tropic acid was not included. However, Gottlieb (6), in his investigations on separating tropic acid from atropic acid by a column partition chromatographic technique, used a specially prepared indicator which locates these acids as blue bands on a red background. In order to detect this alkaloid, as well as its acidic and basic hydrolytic products, an acid-base indicator was wanted that has a range of pH such that it gives a basic reaction for both atropine and tropine and an acidic reaction for tropic acid. In addition the background color must contrast favorably with the colors of the developed spots. Of the conventional acid-base indicators screened, bromphenol blue met these requirements most satisfactorily.[1]

Solutions of atropine alkaloid U. S. P., tropine, and tropic acid were spotted on Whatman No. 1 paper. In the development of the chromatogram as described previously, the upper layer of a *n*-butanol-acetic acid-water (10:1:6) solvent system was used as the developing solution (8). The developed paper was air-dried for one hour and then sprayed with the indicator solution, which was prepared by grinding 10 mg. of bromphenol blue with 1.5 cc. of 0.01 *N* NaOH in a mortar and diluting the mixture to 100 cc. with water.

Qualitative Results.—Atropine and tropine immediately exhibited deep purple spots on a light blue-green background, whereas tropic acid was located as a yellow spot which increased in intensity after a few hours. The *Rf* values were found to be 0.41 for tropine, 0.58 for atropine, and 0.86 for tropic acid. The use of standard reference spots with each analysis of an unknown solution eliminates correction factors for variations in temperature and filter paper.

Quantitative Results.—The chromatogram was scanned as described in the preceding section. The colored spots representing atropine and tropine were scanned at 570 mμ, and that representing tropic acid was scanned at 440 mμ. The total color densities of the spots were directly proportional to the concentrations of the three compounds under consideration over the range 10 to 100 μg. This is illustrated in Table III.

Solutions of completely hydrolyzed atropine (tropine plus tropic acid) were mixed with solutions of atropine base to simulate partially hydrolyzed atropine solutions in the range of 33 to 89% hydrolysis. Table IV shows some results that were obtained in applying the above procedure to the analysis of these solutions.

Recovery of Atropine from Biological Material

Detection of micro amounts of atropine from biological sources has for the most part been conducted by bioassay (9, 10) or by use of radioactive tracer techniques (11, 12). Tonnesen (10), using the Pulewka mouse eye test, estimated that the method gave an accuracy of ± 15%. For establishing a dose-response curve, a complex process involv-

[1] During the completion phase of this study, a method was published describing the use of bromphenol blue for the detection of veratrum alkaloids by paper chromatography (7).

TABLE III.—RELATIONSHIP BETWEEN CONCENTRATION AND TOTAL COLOR DENSITIES OF ATROPINE, TROPINE, AND TROPIC ACID SPOTS

Compound	Concn., μg.	Total Color Density (area under curve in mm.²)	Density/Concn. = K
Tropine	10	15	1.50
	20	31	1.55
	50	80	1.60
	100	162	1.62
		Average	1.57
Atropine	10	11	1.10
	20	21	1.05
	50	54	1.08
	100	106	1.06
		Average	1.07
Tropic acid	10	7	0.70
	20	15	0.75
	50	36	0.72
	100	71	0.71
		Average	0.72

tion removed. The precipitate is resuspended in 5 cc. of 5% TCA and again centrifuged. The supernatant solutions are combined, brought to pH 9 with 10% ammonium hydroxide, and extracted three times with 5-ml. portions of chloroform. The chloroform extracts are combined, washed once with water, then evaporated to dryness under low pressure, using a Rinco rotating vacuum-type evaporator. The residue is taken up in a drop of chloroform, and spotted on the paper in increments of 0.005 ml. The addition of chloroform and the process of spotting are repeated. The chromatographic analysis is then carried out as previously described for atropine and tropine, since this technique has the greater sensitivity. Table V illustrates the results obtained by use of this procedure for analyzing tissues and fluids of young adult white rats that were injected parenterally with atropine. The data on the intravenous injection of atropine sulfate compare favorably with results obtained by Tonnesen (9) which showed that after intravenous injection of 5 mg. into 200-gram rats

TABLE IV.—ANALYSIS OF SOLUTIONS CONTAINING VARYING AMOUNTS OF HYDROLYZED AND UNHYDROLYZED ATROPINE

	Calculated Values				Values by Analysis[a]			
	Contents, μg.			% Hydrolyzed	Contents, μg.			% Hydrolyzed
Solution	Tropine	Tropic Acid	Atropine		Tropine	Tropic Acid	Atropine	
1	19	21	80	33	20	19	77	34
2	39	41	60	57	38	37	58	56
3	48	52	50	67	49	47	51	65
4	58	62	40	75	59	58	43	73
5	78	82	20	89	82	81	19	90

[a] A solution containing 100 μg. each of tropine, tropic acid, and atropine was used as reference spots in this analysis.

ing large numbers of animals, i. e., 10 or more mice per dose, is necessary.

To test the applicability of these chromatographic procedures to biological studies, several methods for extracting micro quantities of atropine from animal tissues were evaluated. A method that gave better than 90% recovery of atropine from various animal tissues and fluids is as follows: To the tissue, 5 cc. of 5% trichloracetic acid (TCA) is added. The mixture is blended in a heavy-walled test tube with a stainless steel Potter-Elvehjem homogenizer. In the analysis of blood or plasma the blending step may be omitted. The mixture is centrifuged for ten minutes at 3000 r. p. m. and the supernatant solu-

atropine disappears rapidly from the blood stream and fairly high concentrations of the alkaloid are found in pulmonary and renal tissue. In our experiment, we also injected the alkaloidal salt, 2.5 mg. into the femoral vein of 200-gram male rats. For comparable studies on the intramuscular injection of atropine in rats, we were unable to find any previous investigations.

SUMMARY

1. A direct microanalytical method for determining atropine and its breakdown product,

TABLE V.—RESULTS OBTAINED IN APPLYING PAPER PARTITION CHROMATOGRAPHY TO THE ANALYSIS OF ATROPINE IN BIOLOGICAL MATERIAL AFTER INJECTION[a]

Type of Injection	Amount of Injection	Time Elapsed After Injection, min.	Micrograms of Atropine per Gram of Wet Tissue		Micrograms of Atropine per cc. of Blood
			Kidneys[b]	Lungs[b]	Blood[c]
i. v.	2.5 mg. $AtSO_4$	1 to 3	..	..	1
i. v.	2.5 mg. $AtSO_4$	10	20	30	.
i. m.	5 mg. $AtSO_4$	3 to 30	..	..	0
i. m.	5 mg. $AtSO_4$	15	0	0	.
i. m.	5 mg. $AtSO_4$	25	10	8	.
i. m.	5 mg. $AtSO_4$	35	4	4	.

[a] All rats used in this experiment gave negative results for the presence of "atropine esterase" on the analysis of their blood (13–16).

[b] Corrected for 92% recovery of 25 μg. of $AtSO_4$ added *in vitro* to tissue as compared to a standard spot containing 25 μg. of $AtSO_4$.

[c] Same correction as for tissue samples except that only 10 μg. of $AtSO_4$ standard was used for comparison.

tropine, has been developed. Concentrations in the range of 2 to 25 micrograms were found to follow Beer's law. A procedure for extracting atropine from biological material has been applied which gives better than 90 per cent recovery.

2. A method using bromphenol blue indicator as a spray reagent has been developed for simultaneously analyzing atropine and its hydrolytic products, tropine and tropic acid, in the range of 10 to 100 micrograms.

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Glutarimides IV.*

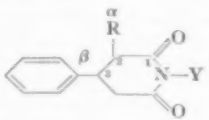
N-Alkyl and N-Aryl Derivatives of 3-Phenylglutarimide and 3-Phenyl-2-Methylglutarimide

By BILLY G. MALLARD and CHARLES O. WILSON†

Derivatives of 3-phenylglutarimide were prepared for pharmacological testing. The investigation was directed toward determining the effect of an aryl group in the *beta* or 3 position as well as on the nitrogen and the effect of a methyl group in the *alpha* or 2 position. The synthesis of glutarimides was accomplished by preparing the corresponding acid, converting this to the anhydride, reacting the anhydride with ammonia or a primary amine, and then heating to split out water and remove the excess ammonia or amine. The 3-phenyl glutarimide derivatives were synthesized by a modified Michael condensation reaction. The 2-methyl-3-phenylglutarimide derivatives were synthesized by the method of Michael and Ross. Eight glutarimide derivatives and ten other intermediate compounds were prepared. The glutarimide derivatives prepared are being investigated pharmacologically.

In previous papers by Benica and Wilson (1, 2) and Mallard and Wilson (3), various derivatives of glutarimide have been reported. The value of a substituent on the nitrogen of a heterocycle (2) and the occurrence of the structural group —CO—NH—CO— in central nervous system depressants (1) have been pointed out. A recent report on the pharmacological activity of 3-ethyl-3-methylglutarimide (4) have given impetus to this particular field of research. This compound possesses properties useful in counteracting barbiturate poisoning (4).

Interest in phenylglutarimides arose from the depressant action of 2-ethyl-2-phenylglutarimide (Doriden®) and N-methyl-3-phenylsuccinimide (Milontin®). In this light it was deemed logical to prepare for pharmacological testing derivatives of 3-phenylglutarimide.



R = H or CH_3
Y = H, alkyl, aryl, or benzyl

The investigation was directed at determining the effect of an aryl group in the *beta* or 3 position as well as on the nitrogen and the effect of a methyl group in the *alpha* or 2 position (Table I).

The synthesis of glutarimides was accomplished by preparing the corresponding acid, converting this to the anhydride, reacting the anhydride with ammonia or a primary amine, and then heating to split out water and remove the excess ammonia or amine. The 3-phenylglutarimide derivatives were synthesized by a Michael con-

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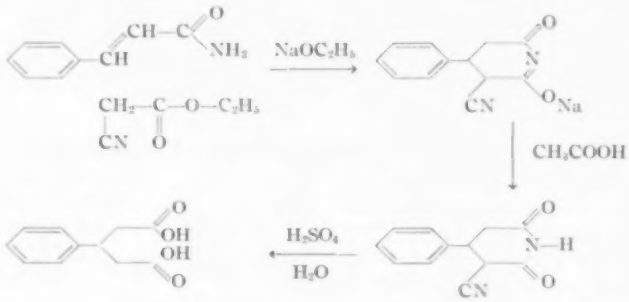
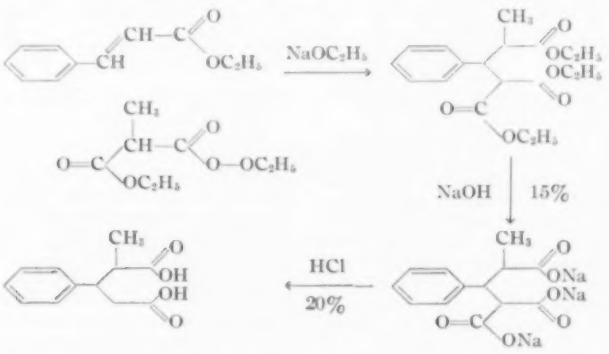
densation as modified by Barat (5) and Connor (6). The condensation product of cinnamamide and ethyl cyanoacetate in sodium ethylate was treated with glacial acetic acid. The imide was converted to the acid with sulfuric acid.



The 2-methyl-3-phenylglutarimide derivatives were synthesized by the Michael and Ross (7) method to give 2-methyl-3-phenyl-4-carboxyglutaric acid followed by acid decarboxylation.



The condensation product of ethyl cinnamate and diethyl methylmalonate in sodium ethylate was treated with sodium hydroxide followed with hydrochloric acid to give the acid. The anhydride of each of the acids was prepared by reacting with acetic anhydride which with ammonia or the appropriate primary amine produced the imide. For analytical data refer to Table I.

Pharmacological investigations are being made of the compounds herein reported.

PROCEDURE[1]

2-Cyano-3-phenylglutarimide.—This compound was prepared by modifying the procedure of Barat (5) and Connor (6) from sodium (23 Gm.), ethyl cyanoacetate (113.2 Gm.), and cinnamamide (147.2 Gm.).

The heavy precipitate of the salt that formed on standing for a week was filtered off with suction, washed with cold absolute alcohol, and dissolved in a minimum amount of water. The aqueous solution was acidified with glacial acetic acid, cooled in an ice bath, and the solid separated by filtration under reduced pressure. The imide was allowed to dry on a porcelain plate at room temperature for forty-eight hours and then dried in a vacuum desiccator over calcium chloride for forty-eight hours. Yield 139 Gm. (65%); m. p. 225–227°. Barat (5) reports a m. p. of 224–225°.

3-Phenylglutaric Acid.—This was obtained by the hydrolysis and decarboxylation method of Barat (5) and Connor (6). 2-Cyano-3-phenylglutarimide (117 Gm.) was treated with concentrated sulfuric acid (400 cc.).

3-Phenylglutaric Anhydride.—3-Phenylglutaric acid (176 Gm.) was dissolved in acetic anhydride (523 Gm.) in a one-liter distilling flask and treated according to the procedure for 3,3-dimethylglutaric anhydride (1).

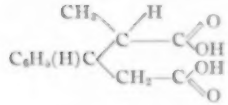
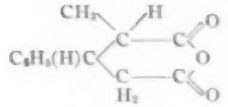
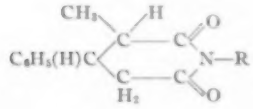
3-Phenylglutarimide.—This was prepared by the method of Mallard and Wilson (3).

N-Methyl-3-phenylglutarimide.—3-Phenylglutaric anhydride (9.5 Gm.) was dissolved in xylene 25 cc. and treated with methylamine according to the method of Mallard and Wilson (3).

N-Phenyl-3-phenylglutarimide.—3-Phenylglutaric anhydride (9.5 Gm.) was dissolved in xylene (15 cc.) and added to a solution of aniline (4.6 Gm.)

[1] Melting points are uncorrected.

TABLE I.—ANALYTICAL DATA ON COMPOUNDS STUDIED

Compound	Formula	M. p.	Yield, %	Carbon		Hydrogen	
				Calcd., %	Found, %	Calcd., %	Found, %
$C_6H_5(H)C(CH_2COOH)_2$	$C_{11}H_{12}O_4$	138–139°	72	63.45	63.63	5.77	5.78
$C_6H_5(H)C(CH_2CO)_2O$	$C_{11}H_{10}O_3$	105–106°[a]	82	69.45	69.35	5.26	5.40
$C_6H_5(H)C(CH_2CO)_2N—R$							
R = H—	$C_{11}H_{11}NO_2$	173.5–174.5°	62.7	69.29	69.66	5.86	6.00
CH_3—	$C_{12}H_{13}NO_2$	141–142°	75.1	70.91	70.91	6.30	6.60
C_6H_5—	$C_{17}H_{15}NO_2$	222–223°	63.4	76.80	76.68	5.73	5.67
$C_6H_5CH_2$—[e]	$C_{18}H_{17}NO_2$	100–101°[b]	31.9	77.40	77.34	6.09	5.86
	$C_{12}H_{14}O_4$	103–109°[c]	87.4	64.80	64.89	6.31	6.53
	$C_{12}H_{12}O_3$	200–205°/ 8 mm.[d]	84.4	70.57	70.67	5.92	6.18
							
R = H—	$C_{12}H_{13}NO_2$	136–137°	70.4	70.91	71.21	6.30	6.75
CH_3—	$C_{13}H_{15}NO_2$	111–112°	74.0	71.49	71.50	6.96	6.88
C_6H_5—	$C_{18}H_{17}NO_2$	180–182°	60.4	77.39	77.26	6.13	6.01
$C_6H_5CH_2$—	$C_{19}H_{19}NO_2$	93–94°	54.6	77.80	77.61	6.49	6.53

[a] Reported 105° (8 and 9). [b] Reported 98–99° (10). [c] Reported 125° (12). [d] B. P.; wax-like solid. [e] N-Calcd. 5.02%; found 4.93%.

in xylene (15 cc.) in a 125-cc. flask and treated according to the method of Mallard and Wilson (3).

N - Benzyl - 3 - phenylglutarimide.—Benzylamine (2.7 Gm.) was dissolved in 15 cc. xylene and added to a solution of 3-phenylglutaric anhydride (4.8 Gm.) in 15 cc. of xylene and treated according to the method of Mallard and Wilson (3).

The product was distilled and the imide collected at 245–250°/8 mm. The distillate solidified and was recrystallized from hot alcohol.

Triethyl-2-methyl-3-phenyl-4-carboxyglutarate.—This was prepared by the method of Michael and Ross (7). Diethyl methylmalonate (11) (67 Gm.) was collected at 77–78°/8 mm. Ethyl cinnamate (74 Gm.) was collected at 121–122°/7 mm. Triethyl α-methyl-β-phenyl-α'-carboxy glutarate was collected at 188–191°/6 mm. Michael and Ross (7) have reported a boiling point of 194–195°/3 mm. Yield 301 Gm. (64.8%).

2-Methyl-3-phenyl-4-carboxyglutaric Acid.—Triethyl-2-methyl-3-phenyl-4-carboxyglutarate (120 Gm.) was dissolved in 1260 cc. of 10% sodium hydroxide in 50% alcohol and the procedure of Michael and Ross (7) followed. Yield 80 Gm. (86.1%); m. p. 167–168° with evolution of gas. Michael and Ross (7) reported 171°.

2-Methyl-3-phenylglutaric Acid.—2-Methyl-3-phenyl-4-carboxyglutaric acid (78 Gm.) was placed in a one-liter round bottom flask containing 200 cc. of 20% hydrochloric acid. The mixture was heated under reflux for twenty-four hours and then allowed to cool. An oil separated which crystallized upon standing. The solid was separated from the aqueous portion, broken up, and dried on a porous plate.

The product was recrystallized by dissolving in benzene and precipitating by the addition of petroleum ether, m. p. 103–109°.

2-Methyl-3-phenylglutaric Anhydride.—2-Methyl-3-phenylglutaric acid (120 Gm.) was dissolved in acetic anhydride (330 Gm.) and treated according to the procedure for 3,3-dimethylglutaric anhydride (1).

There were eight glutarimide derivatives prepared (Table I) and ten other intermediate compounds.

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An Approach to Antacid Evaluation*

By ROBERT H. SCHLEIF

An apparatus and a procedure were set up for the evaluation of antacids. This evaluation was carried out simulating conditions as they may be found in a stomach in the hyperchlorhydric state. Data and figures presented show speed of action, maximum pH reached, and duration of action for several compounds having antacid properties and for several proprietary antacid products. In most instances reproducibility of results was excellent with only two determinations. It is suggested that this method may well serve as standard procedure for the evaluation of antacids.

NUMEROUS METHODS have been advanced for the evaluation of antacids (1–11). These methods may be divided into three groups. In the first group are those procedures in which simulated gastric juice or 0.1 *N* HCl is periodically added to an initial quantity of simulated gastric juice and antacid. The pH is recorded at various intervals (1, 5–8). In the second group are those in which not only is 0.1 *N* HCl or simulated gastric juice added at frequent intervals, but some of the solution is also removed from the beaker to simulate emptying of the stomach (4). In the third group are those methods employing the continuous flow of 0.1 *N* HCl and continuous recording of the pH after the antacid sample had been added to an initial quantity of 0.1 *N* HCl (11, 9, 10). An evaluation of procedures in the first two groups was recently reported by Booth and Dale (12). They found no apparent correlation between the various methods, and they recommended that a standard procedure be set up for the evaluation of antacids, since one antacid when tested by one method would be considered poor and with a second method it would be considered very satisfactory.

In order that a given procedure be considered as a standard method it should have certain criteria. First, the method should show speed of action, maximum pH reached, and duration of action. Secondly, the procedure should correlate with *in vivo* studies, and thirdly, the method should have good reproducibility.

Rossett and Flexner (10) made a comparison between an *in vitro* method involving continuous flow of 0.1 *N* HCl and continuous recording of pH with an *in vivo* method in which electrodes connected to a recording pH meter were placed in the human stomach and recordings of the effects of antacids made. The authors found that the *in vitro* method produced results which were quite similar to those found with the *in vivo* method. Since this procedure most closely fits the criteria set forth in the preceding paragraph, it was decided to use a procedure and an apparatus similar to those of Rossett and Flexner for this study.

EXPERIMENTAL

The apparatus shown in Fig. 1 consists of a Beckman Model W pH meter, a Minneapolis-Honeywell strip chart recorder, a Brewer automatic pipet, a magnetic stirrer placed beneath a water bath and heating unit, a solenoid trip mechanism, and timers. These were built into a panel unit.

All tests were made at approximately 37°. Agitation was carried out at a constant rate with a magnetic stirrer. Simulated Gastric Juice U. S. P. XV was used. One-gram samples of the antacid compounds were used. Except for 3 cases indicated in Table II, 2 crushed tablets were used in the test of products.

Fifty milliliters of the simulated gastric juice and 50 ml. of distilled water were placed in a beaker in the bath and allowed to come up to proper temperature. The sample to be tested was placed on a platform above the beaker. The trip mechanism was released and the sample dropped into the beaker. At the same time a timer was automatically activated, and fifty seconds later the timer activated the automatic pipet and the simulated gastric juice began to flow into the beaker at the rate of 0.4 ml. per stroke with 10 strokes per minute. The test period was from one to two hours, dependent on the apparent duration of the action of the antacid.

Speed of action was designated as the time interval from the start of the test until pH 3 was reached. Duration of action was designated as the time interval from the start of the test until the pH of the solution returned to 2.

The records shown in Figs. 2 to 9 were photographed in reverse in order to read the curves from left to right. Time is the abscissa with each space representing ten minutes, and pH is the ordinate as shown.

DISCUSSION

The rate of flow indicated above was chosen for the reason that Rossett and Rice (11) found this rate most closely simulated conditions which obtain in the hyperchlorhydric state in a duodenal ulcer case. According to Fuchs and others (8, 10), 2 ml. per

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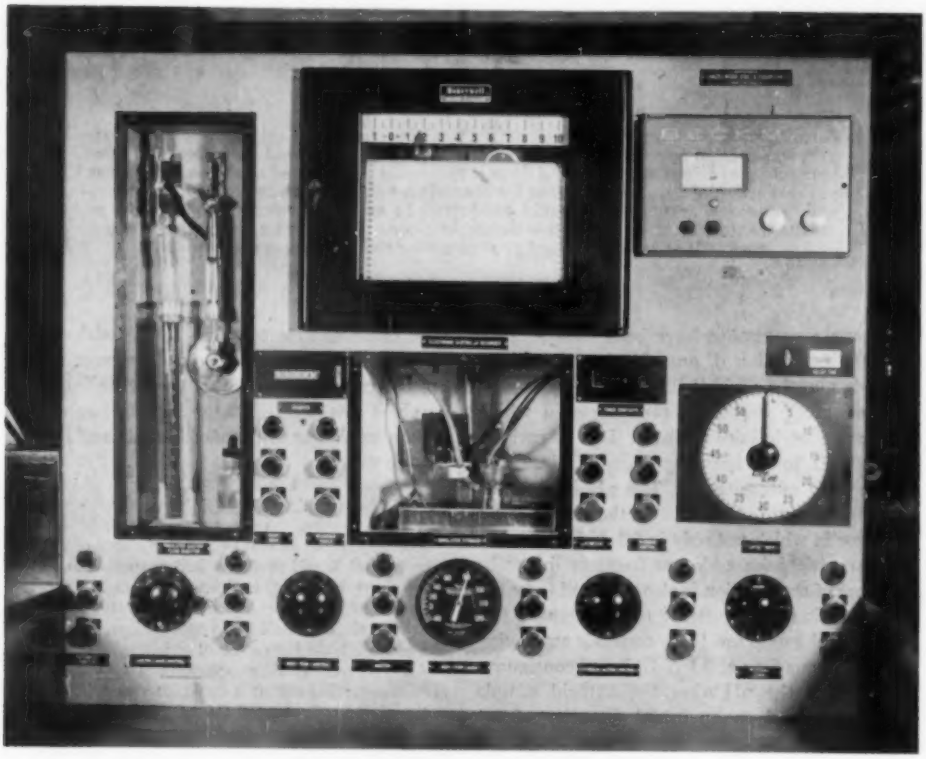

Fig. 1.—Photograph of apparatus.

minute appears to be the approximate quantity secreted in the hyperacid condition not accompanied by a duodenal ulcer. According to this, the rate actually used in these tests would represent an extreme situation; nevertheless, the rate was considered desirable, since it cut the testing period from two to four hours down to one to two hours. The use of an initial quantity of 50 ml. of simulated gastric juice was based on a calculation by Fuchs (8) that approximately 50 ml. of free 0.1 *N* HCl was present in the stomach one-half hour after meals.

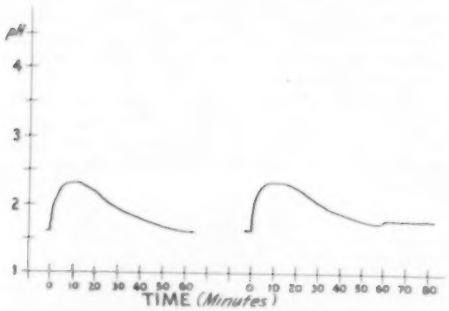

Fig. 2.—Record of two determinations made with 1-Gm. samples of magnesium trisilicate.

Reproducibility.—With few exceptions the tests were reproducible, and in practically all instances only two determinations were needed. This was true for both the antacid compounds and the antacid products. Illustrations of the reproducibility of the method are shown in Figs. 2 to 9. Records

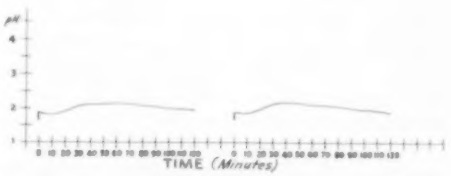

Fig. 3.—Record of two determinations made with 1-Gm. samples of dried aluminum hydroxide gel.

of 2 or 3 determinations are shown side by side. It may be seen that both curves for the various substances tested are very similar to each other. In the few instances in which poor reproducibiiity was obtained it was found to be due to poor mixing. When this situation was rectified good results were obtained.

Speed of Action.—The time required for the solution to reach pH 3 was taken as an indication of

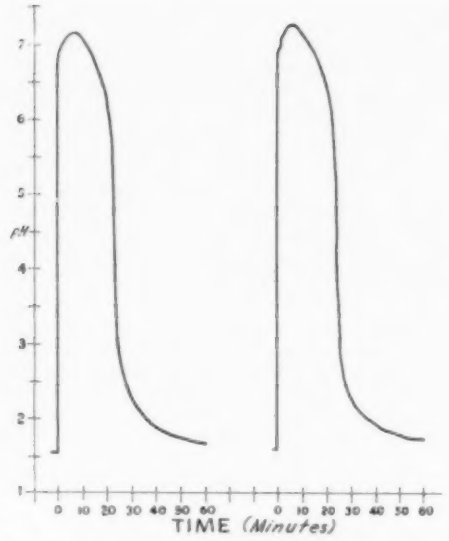

Fig. 4.—Record of two determinations made with 1-Gm. samples of sodium bicarbonate.

speed of action, since at this pH the free hydrochloric acid is considered to be neutralized (13, 14). It may be seen in Table I that magnesium trisilicate and dried aluminum hydroxide gel did not raise the pH to 3 during the test.

If pH 2 were arbitrarily taken as the point to determine speed of action, the speed of action would be approximately one minute for magnesium trisilicate (Fig. 2) and approximately twenty-three minutes for dried aluminum hydroxide gel (Fig. 3). For the other compounds studied the speed of action did not differ to a significant degree. In Table II it may be seen that products G, I, and M did not raise the pH to 3. If pH 2 were taken as the point to determine speed of action, product G (Fig. 7) would have a speed of action of five minutes, and product M one of approximately three minutes. Product I raised the pH to only 1.85, but an inspection of the

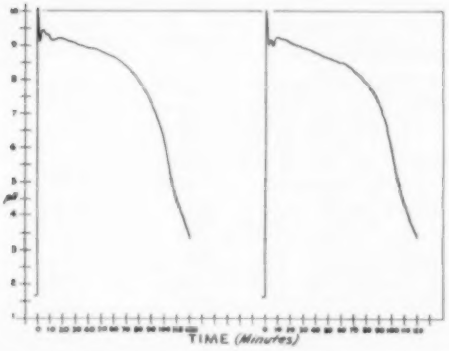

Fig. 5.—Record of two determinations made with 1-Gm. samples of magnesium oxide.

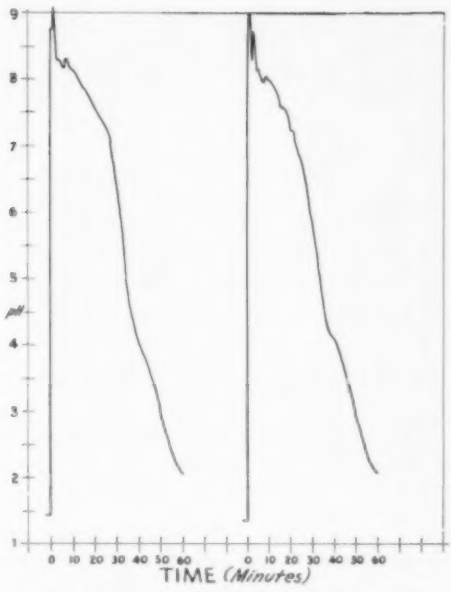

Fig. 6.—Record of two determinations made with samples of 2 crushed tablets containing magnesium hydroxide (Product J).

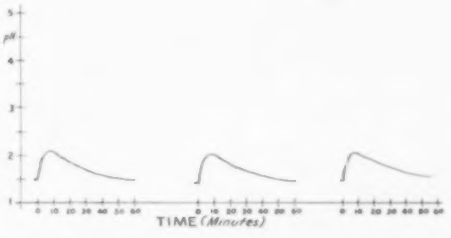

Fig. 7.—Record of three determinations made with samples of 2 crushed tablets containing aluminum hydroxide (Product G).

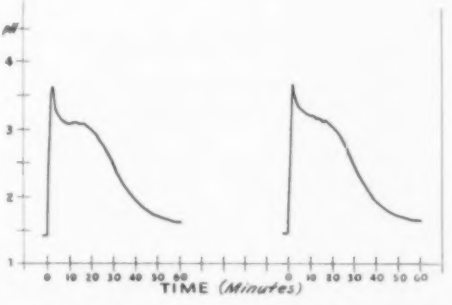

Fig. 8.—Record of two determinations made with samples of 2 crushed tablets containing dihydroxy aluminum sodium carbonate (Product B).

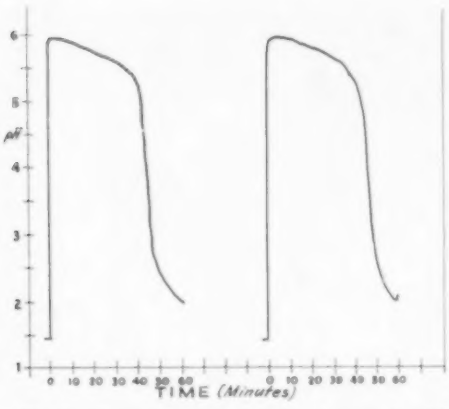

Fig. 9.—Record of two determinations made with samples of 2 crushed tablets containing calcium carbonate, magnesium trisilicate, and magnesium carbonate (Product A).

would mean that free hydrochloric acid should again be present. On this basis the point to choose as the end point of action of a given antacid should be pH 3, but many of the antacids exert their action around pH 3 or below; therefore it was considered more convenient to choose pH 2 as the end point. Only product I did not reach pH 2. This made it possible to compare all products and compounds on the same basis. It would appear that a procedure such as the one used in this study, in which a continuous flow of simulated gastric juice is used, would provide a more reliable picture of the duration of action as it might be in the stomach than when acid is added at various intervals. Data on duration of action are shown in Tables I and II.

Maximum pH.—The figures listed in Tables I and II are the maximum pH values attained by the various compounds and products under the conditions of the test. Some of these values are lower than those often stated in the literature, but generally the values are in the range which one would expect for these substances. The lower figures are probably due to the fact that in this procedure the flow

TABLE I.—AVERAGE VALUES OBTAINED WITH ANTACID COMPOUNDS

Antacid Compound	Speed of Action, sec., to pH 3	Duration of Action, min., to pH 2	Maximum pH	Total ml. Simulated Gastric Juice
Calcium carbonate	3.2	73	6.37	338
Magnesium trisilicate	...[a]	28	2.3	162
Magnesium carbonate	5.4	75	7.8	350
Sodium citrate	11.3	34	4.85	187
Sodium bicarbonate	3.0	39	7.25	208
Magnesium oxide	4.8	128	9.9	587
Dihydroxy Al Na carbonate	13.5	88	4.15	412
Dried alum. hydroxide gel	...[a]	98	2.15	456
Dihydroxy Al aminoacetate	28.8	94	4.1	441

[a] Did not go up to pH 3.

shape of the curve indicated that the speed of action was approximately ten minutes.

Duration of Action.—Since pH 3 is considered as the point at which the free hydrochloric acid in the stomach has been neutralized, a return to just below that pH, after the pH has been above that point, of simulated gastric juice was continuous and also carried out at a faster rate.

Volume of Simulated Gastric Juice Used.—The last columns in Tables I and II list the quantities of simulated gastric juice which flowed into the beaker from the beginning of each determination until the

TABLE II.—AVERAGE VALUES OBTAINED WITH ANTACID PRODUCTS

	Product Contents	Speed of Action, sec., to pH 3	Duration of Action, min., to pH 2	Maximum pH	Total ml. Simulated Gastric Juice
A	Calcium carbonate, mag. carbonate, mag. trisilicate	5.0	60	5.9	290
B	Dihydroxy alum. sod. carbonate	41.8	39	3.65	206
C	Calcium carbonate, glycine	9.7	46	5.57	234
D	Mag. trisilicate, calcium carbonate, mag. hydroxide	15.0	38	6.75	203
E	Sodium bicarbonate, citric acid	4.0	66	5.85	314
F	Sodium bicarbonate, citric acid	3.5	66	5.13	318
G	Aluminum hydroxide	...[a]	11	2.07	95
H	Aluminum hydroxide, mag. trisilicate	261.0	29	3.15	166
I	Aluminum hydroxide	...[a]	80[b]	1.82	370
J	Magnesium hydroxide	4.4	63	9.02	304
K	Alum. hydroxide, calcium carbonate	82.0	25	4.3	152
L	Alum. hydroxide, mag. hydroxide	81.0	13	3.3	103
M	Hydroxy alum. mag. aminoacetate	...[a]	43	2.75	226

[a] Did not go up to pH 3. [b] Did not reach pH 2—pH 1.5 taken as end point. Product E—1 crushed tablet used. Product F—4.1 Gm. granules used. Product K—2 whole tablets used.

pH had returned to 2. The values also include the 50 ml. of juice placed in the beaker before each determination was begun. Except in a few instances these values are not a measure of the actual amount of simulated gastric juice neutralized by the 1-Gm. samples of antacid compounds or product samples, but more correctly represent a volume of simulated gastric juice which could be handled by the antacid and still maintain the pH above 2.

Since the rate of flow of the simulated gastric juice is known, and since pH is plotted against time on the records, it is possible to determine from the curves the amount of simulated gastric juice which has flowed into the beaker of solution at any pH level, and the test is not confined to any one pH value.

A comparison was made between the results of this study and the work of Rossett and Flexner (10). Although comparisons could only be made with a few compounds, in those cases in which it was possible similar results were obtained.

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The Nature of Scandol*

By ELIAS J. COREY, STEPHEN PROSKOW, and LLOYD M. PARKS†

Scandol, $C_{30}H_{50}O$, from *Celastrus scandens* Linné has been shown to be a mixture containing lupeol, $C_{30}H_{50}O$, and β-amyrin, $C_{30}H_{50}O$, as the major constituents.

THE WIDE DISTRIBUTION of triterpenes in nature and their structural and biosynthetic relationship to steroids have of late attracted increasing attention from workers in the field of organic natural products (1). The known triterpenes (all C_{30} compounds) may be classified into several groups according to the number of carbocyclic rings and the structure of the carbon atom framework. The individual members of each group are distinguished by the number, location, and type of oxygen substituents and may be regarded as oxygenated derivatives of a parent compound of formula $C_{30}H_{50}$ or $C_{30}H_{50}O$. Each of the parent members appears to be derived biosynthetically from squalene ($C_{30}H_{50}$), the fundamental, acyclic hexaene containing six isoprene units, by cyclization, with or without accompanying rearrangements. The remaining triterpenes are produced from the parents by oxidation.

In the tetracyclic and pentacyclic series, which are the most frequently encountered, the least oxygenated naturally occurring members are not the hydrocarbons, but the 3-monohydroxylated derivatives and for this reason it is convenient to regard these $C_{30}H_{50}O$ compounds as parent substances. The most important group parents of the tetracyclic series are the substances lanosterol, $C_{30}H_{50}O$, (1*c*), euphol, $C_{30}H_{50}O$, (1*c*, 2) and tirucallol, $C_{30}H_{50}O$, (3). In the pentacyclic series the important parent members (all $C_{30}H_{50}O$) include α-amyrin (4), β-amyrin (5), lupeol (6), taraxasterol (7), taraxarol (8) and friedelin (9). It is attractive to the organic chemist to envisage the biosynthesis of these pentacyclic triterpenes not only as commencing from the common precursor, squalene, but also as proceeding via a common intermediate, the lupanyl cation (10) by a series of 1,2-shifts of carbon and hydrogen of the type so common in the terpene series. The $C_{30}H_{50}O$ triterpenes are, therefore, of great interest.

This knowledge made it imperative to examine further the material scandol, $C_{30}H_{50}O$, which was obtained from *Celastrus scandens* Linné and reported on briefly twelve years ago (11, 12) as a possible parent of a new group of triterpenes. The material isolated in the original work gave

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analytical results in close agreement with the formula $C_{30}H_{50}O$ and was shown to possess one double bond (peracid titration) and a hydroxyl group (formation of acetate and benzoate derivatives) (12). It also appeared to be homogeneous as judged at the time from recrystallization and melting point experiments. β-Amyrin and lupeol were also obtained during the isolation of scandol. If these data were correct, scandol would have to be a new kind of pentacyclic triterpene.

The same material has now been investigated using newer techniques and has been shown not to be a pure substance, but a mixture. The first samples studied were those described in Table II of reference 12 as fractions 4, 5, and 6 in which β-amyrin, lupeol, and scandol were originally found. The material was acetylated and recrystallized to yield pure β-amyrin acetate and a mixture of acetates. The mixture of acetates upon chromatography afforded pure lupeol acetate and a series of fractions which were mixtures, but no scandol acetate. The mixture fractions were combined, saponified, and subjected to countercurrent distribution, but none of the fractions yielded homogeneous material with the reported physical constants of scandol.

Further experiments on a sample of "purified scandol," m. p. 163.2–166°, which had been obtained in the original investigation (12), led to the resolution of the major part of this material (as acetate and benzoate esters) into β-amyrin and lupeol. Quantitative infrared analysis on this scandol sample showed that the material contained *ca.* 35 per cent lupeol (analysis at 885 $cm.^{-1}$ where lupeol displays interference-free $C{=}CH_2$ absorption). The infrared spectrum showed no bands which do not appear in the spectra of β-amyrin and lupeol. In addition the optical rotation of this sample of scandol, $[\alpha]_D^{25°}$ + 56.9°, is midway between that of β-amyrin (+88°) and lupeol (+27°). This sample of scandol melted quite sharply, however, and was not affected appreciably by recrystallization.

Although it was not possible to account for all the supposedly pure scandol as β-amyrin and lupeol, it is clear that the previously described material is largely a mixture of these two triterpenes and not a pure substance. It is advisable, therefore, that the name scandol be dropped as inappropriate for the material in question.

EXPERIMENTAL

All melting points have been corrected and were taken on the Kofler micro-block unless otherwise noted.

Preliminary Investigation.—β-Amyrin acetate and lupeol benzoate were readily isolated from a portion of fraction 6 (Table II, reference 12) by the procedure of Cook, *et al.* (12), but scandol benzoate could not be obtained by repeated recrystallization of the remaining material. Suitable solvents were butanol-1 and a mixture of butanone-95% ethanol.

Fractionation of Crude Scandol.—A 1.0-Gm. portion of fraction 6 was acetylated by refluxing with a mixture of acetic acid-acetic anhydride (5 ml., 3 ml.) for three hours, and 0.63 Gm. of crystals, m. p. 183–215° (cap.), were removed by filtering the cooled reaction mixture. When recrystallized thrice from boiling acetic acid-acetic anhydride (about 4:1), these gave pure β-amyrin acetate, m. p. 240–242° (cap.). The remaining acetylated material was then isolated by vacuum—evaporating the first filtrate above.

This crude acetate [0.41 Gm., m. p. 163–215° (cap.)], was chromatographed on a column of neutral grade alumina (18.0 Gm., ht. 340 mm.). *n*-Hexane was used as solvent and eluent; 25-ml. fractions of eluate were collected. Fractions 5–7 inclusive each gave crystals of m. p. about 170–195°. These fractions were combined (60 mg.), and recrystallized once from acetic acid-acetic anhydride, giving long needles, m. p. 190–210°. Fractions 8–10 had m. p.'s 158–194°, 162–168°, and 160–176° respectively. These were combined (total wt. 9 mg.) and examined for scandol acetate [reported (12) m. p. 165–168°]. One recrystallization from absolute ethanol gave crystals of m. p. 171–214°. Fractions 11–19 contained nearly all of the remaining material. The m. p.'s of the individual fractions were high and indicative of lupeol acetate. The combined material, recrystallized twice from absolute ethanol, gave pure lupeol acetate, m. p. 220–222°.

Finally, the combined fractions 5–10 were rechromatographed as above. The fractions collected had m. p.'s ranging from 173–198° to 204–210°, but no individual fraction had a m. p. lower than 170°.

Countercurrent Distribution.—Large scale investigations were carried out on the combined fractions 4 and 5 (10.5 Gm., 16.1 Gm.). This was acetylated by the pyridine-acetic anhydride method to remove two crops of β-amyrin as the acetate. A total of 15.2 Gm. of β-amyrin acetate, m. p. 230–243°, was obtained.

The remaining acetate mixture (11.0 Gm.) was then chromatographed by the procedure described in the previous section. In this experiment acid-washed alumina (350 Gm., 30 × 560 mm.) was used. A lupeol acetate-rich portion was removed and discarded (about 1.0 Gm.); the fractions which showed promise of scandol acetate again gave rise to high melting products when repeatedly recrystallized from 95% ethanol and petroleum ether (b. p. 40–60°).

With the exception of the discarded lupeol acetate, all the chromatography fractions were combined and saponified in the usual way. The crude alcohol mixture (8.3 Gm.) was then run through an all glass countercurrent distributor (80 ml. machine, 54 plates). The solvent system, *n*-hexane and 85% ethanol, showed a partition coefficient of about 0.25 for the crude material. It was found that all of the material could be accounted for in the first 33 transfers of the withdrawn series, and that the distribu-

tion curve was like that of a pure compound. Individual fractions when recrystallized from methanol-acetone (1:1) did not give any scandol. By recrystallizations of the benzoate, it was shown that appreciable quantities of lupeol were still present in the mixture.

Investigations with Authentic Purified Scandol and Derivatives.—Samples of scandol, scandol acetate, and scandol benzoate from the work of reference 12 were examined. With the Kofler micro-block, we found that the m. p. of scandol was 163.2–166°, but the acetate and benzoate behaved as mixtures. Thus, about three-fourths of a sample of the acetate melted at 170–172°, most of the remaining crystals at 178°, and some needles did not melt until 215°. The benzoate softened at 197°, melted mostly at 210–219°, with last crystals disappearing at 235°. These results suggest that scandol represents a eutectic mixture, which when acetylated or benzoylated gives products no longer having a eutectic composition. Since the sample of scandol has $[\alpha]_D^{25}$ + 56.9° and the optical rotations of β-amyrin and lupeol in the same solvents are reported to be $[\alpha]_D$ + 88° and $[\alpha]_D$ + 27.2°, it was presumed to consist of a 50:50 mixture of these components.

The presence of β-amyrin was confirmed via acetylation of 15 mg. of the scandol sample. The crude product, m. p. 171–187°, when recrystallized once from acetic acid-acetic anhydride, gave long needles (3.5 mg.) consisting of β-amyrin acetate, m. p. 219–232°.

The presence of lupeol was shown by comparing the infrared spectra of lupeol acetate and β-amyrin acetate with that of authentic scandol acetate. Maxima at 1640 cm.$^{-1}$ and 885 cm.$^{-1}$ confirmed the presence of lupeol, and all bands in the spectrum of the scandol acetate could be explained by summing up the spectra of β-amyrin and lupeol. The spectrum of lupeol exhibits a sharp band at 885 cm.$^{-1}$ (ϵ = 161 mg./cm.^{2}) which is absent in the spectra of other triterpenes lacking C=CH_2. On this basis and from the spectrum of scandol acetate the lupeol content of scandol acetate was calculated to be 35 ± 5%.

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Preliminary Investigation of the Fungistatic Properties and Toxicity of Certain Resorcinol Derivatives*

By FRANK W. ZABRANSKY† and HAROLD N. WRIGHT‡

Eight resorcinol derivatives, four monosubstituted, four disubstituted, have been compared for fungistatic activity against *Candida albicans* and *Microsporum gypseum*, for acute and subacute toxicity by intraperitoneal injection, and for local irritant properties.

THE BACTERIOSTATIC and bactericidal properties of resorcinol and various resorcinol derivatives are comparatively well established, and these compounds have been used in the prevention and treatment of bacterial infections of the skin and mucous membranes for a considerable number of years. For instance, Klarman, Gates, and Shternov (1) studied the bactericidal properties of a fairly large group of alkyl and aryl resorcinol monoethers, and found that they all possessed appreciable bactericidal properties and concluded that in the resorcinol series only one free hydroxyl group was essential for bactericidal activity.

Much less, however, is known of the fungistatic and fungicidal properties of these compounds. Resorcinol was recommended for clinical use as fungicide by Brown (2) in 1933. Resorcinol monoacetate (Euresol)® has been listed in New and Nonofficial Remedies since the inception of that reference work in 1909, with the statement that it "is used as an adjuvant in the treatment of acne, of sycosis vulgaris, of alopecia and of seborrhea," but the manufacturers of Euresol stated in 1955 that "no studies regarding its fungicidal properties are available, though it is a very old product" (3). Woodward, Kingery, and Williams (4),

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in 1933, reported that several resorcinol monoethers possessed fungicidal properties.

Because of the lack of specific information in regard to them, it appeared desirable to investigate the antifungal properties of a group of related resorcinol derivatives. The group of eight compounds[1] investigated in this present report provides a comparison of resorcinol diacetate with resorcinol monoacetate, of resorcinol dimethyl ether with the monomethyl ether, and the dibenzoate with the monobenzoate. The two other derivatives tested were resorcinol diacetic acid and 4-chlororesorcinol, this latter compound being the only one representing a substitution of a hydrogen atom of the benzene ring.

METHODS AND MATERIALS

The compounds were tested for fungistatic activity, using a modification of the cylinder-plate method of Hillegas and Camp (5). Cylinders of stainless steel were used. Because of the known lag period encountered in the growth of newly transferred cultures, four hours were allowed to elapse between inoculation of the plates with the test organism and the application of the test compound. A commercial antifungal preparation, Desenex Solution Undecylenic Acid 10% N. N. R.[2] (a 10% solution of undecylenic acid in propyl alcohol, propylene glycol, and water) was used as the reference compound. The compounds tested were prepared as 10, 1, 0.1, and 0.01 per cent solutions in 95% methyl alcohol, and 95% methyl alcohol was used as the control. Methyl alcohol was employed as the solvent and control because all of the resorcinol derivatives were sufficiently soluble in it; it was less complex than the solvent of the commercial reference preparation and none of the aliphatic alcohols are known to possess antifungal properties.

More than a dozen pathogenic fungi and yeast-like organisms have been used by various workers in this field and there appears to be little uniformity in their choice of organisms. The organisms used[3] for making the tests of fungistasis were *Candida albicans* (*Monilia albicans*) and *Microsporum gypseum*. *Candida albicans* is a yeast-like organism which is very prevalent and which may cause infections of the mouth (thrush), the vagina, the skin of the axilla, inguinal region, hands, and feet (athlete's foot), as well as systemic and generalized infections of the lungs, lymph nodes, liver, spleen, and brain. *Microsporum gypseum* is one of the group of organisms that causes ringworm of the scalp, and may also cause inflammatory reactions resembling a pyoderma. These two organisms were chosen as representative both of different forms of pathogenicity and of cultural characteristics.

In preparing the agar plates a suspension of a 3-day old culture of *C. albicans* was prepared in 4–10 ml. of normal saline to produce a milky appearance, and 0.2-ml. portions of this suspension were delivered with a pipet into petri plates and thoroughly mixed with 20 ml. of Sabouraud's dextrose agar. The procedure with *M. gypseum* differed only in the manner of dislodging the spores from the stock culture, which was accomplished by flooding the surface of the culture with normal saline solution and adding 5 drops of Tween® 80 to aid the solution in permeating the filamentous material.

Acute toxicity was determined in mice (ABC and ZBC) of both sexes by intraperitoneal injection, using aqueous solutions or suspensions. A total of 127 mice was used in the acute toxicity tests, averaging 14 mice per compound tested. The 95 per cent confidence limits of the acute LD_{50} doses were calculated by the method of Litchfield and Wilcoxon (6). Subacute toxicity was determined by intraperitoneal injection of 20, 40, and 80% of the previously determined LD_{50} into mice on each of five consecutive days. Records of survival in both series were maintained for 30 days. Irritation tests were made by the rabbit conjunctival reaction test employing 5% suspensions in 10% acacia. The reaction was observed immediately after instillation and 12, 24, 48, and 72 hours later. An equivalent amount of 10% acacia was instilled into the other eye which was used as a control.

EXPERIMENTAL

The experimental results are summarized in Table I. In the tests of fungistatic activity the plates were read 72 hours after inoculation. Zones of inhibition were read in millimeters, and the table shows the range of values obtained in three series of tests.

Inspection of the table indicates clearly that all the fungistatically active resorcinol compounds, as well as the chemically unrelated undecylenic acid, were more active against *M. gypseum* than against *C. albicans*. This is in agreement with the known clinical difficulty of treatment of *C. albicans* infections (7).

Structurally it is interesting that all three of the monosubstituted resorcinols, as well as the ring-substituted 4-chloro- derivative, were fungistatically active, whereas only one of the four disubstituted resorcinols, namely the diacetate, showed any evidence of fungistatic activity. These results confirm the findings of Woodward, *et al.* (4), that in general, at least one free hydroxyl group must be present in the resorcinol series in order to have antifungal activity.

Three of the four mono- or ring-substituted compounds were as fungistatic as undecylenic acid against both organisms and the one slightly weaker compound, the monobenzoate, was fungistatic over a wider range of concentrations. Two of the four compounds, 4-chlororesorcinol and the monomethyl ether are, however, probably ruled out of clinical usefulness by their irritant properties. Of the remaining two, resorcinol monoacetate is already in clinical use as an antifungal agent. However, resorcinol monobenzoate would appear to be worthy of more extensive investigation as a possibly useful antifungal agent, in view of its apparent lack of irritant properties and its possession of fungistatic activity over a wide range of concentrations, being

[1] Supplied through the courtesy of Koppers Company, Inc., Pittsburgh, Pa.

[2] Manufactured by Wallace and Tiernan, Inc., Belleville 9, N. J.

[3] The cultures of these organisms were obtained through the courtesy of the Department of Bacteriology and Immunology of the University of Minnesota Medical School.

TABLE I.—FUNGISTATIC ACTIVITY, CONJUNCTIVAL IRRITATION AND TOXICITY OF VARIOUS RESORCINOL DERIVATIVES

	Fungistatic Activity							Toxicity			
	M. gypseum			*C. albicans*				Acute I. P.		Subacute[c]	
Compound	10% mm.[a]	1% mm.	0.1% mm.	10% mm.	1% mm.	0.1% mm.	Conj. Irrit.[b]	LD_{50} mg./Kg.	19/20 lim. mg./Kg.	40% LD_{50} D/T	80% LD_{50} D/T
Resorcinol	...	...	...	...	...	...	...	240	189–305	0/4	1/4
4-chlororesorcinol	55–80	40	0	27–30	0–21	4–25	±	195	172–216	0/4	0/4
Res. monometh. ether	70–90	30	0	30	0–14	0	+++	320	260–395	0/4	4/4
Res. monoacetate	58–63	0–12	0	15–20	0	0	++++	400	380–420	0/4	6/8
Res. monobenzoate	17–35	20–30	27	15–20	15	12	±	710	600–835	0/4	3/4
Res. dimeth. ether	0	0	0	0	0	0	0	900	732–1105	0/4	3/4
Res. diacetate	40–60	20–25	0	16–18	0–12	0	+++	660	435–1010	0/4	4/4
Res. dibenzoate	0	0	0	0	0	0	0	8000	...	0/4	0/4
Res. diacetic acid	0	0	0	0	0	0	0	540	470–620	1/4	4/4
Undecylenic acid	40–60	0–45	0	30	0–20	0–10	0	...	...	...	...
Controls	0	0	0	0	0	0	0	...	...	...	...

[a] Range of mean diameter of zone of inhibition 72 hours after inoculation.
[b] Extent of conjunctival irritation: ± mild hyperemia; +++ severe hyperemia, edema of nictitating membrane and eyelid; ++++ same as +++, plus clouding of cornea.
[c] Stated per cent of LD_{50} dose given daily for 5 days. Animals observed for 30 days.

the only compound of the group that showed any fungistatic activity at 0.01% concentration.

The toxicity test showed a fairly wide range of variation in acute toxicity among the various resorcinol compounds tested. Resorcinol and 4-chlororesorcinol were of approximately the same toxicity, while the monomethyl ether and monoacetate were somewhat less toxic. Next in order of decreasing toxicity were the diacetic acid derivative, the diacetate, the monobenzoate and dimethyl ether. The extreme lack of toxicity of the dibenzoate was probably largely due to its poor solubility. All of the compounds produced toxic symptoms similar to those that have been described for resorcinol (8), namely, irritability, tremors, ataxia, salivation, lachrymation, piloerection, and convulsions, with terminal depression.

In the subacute toxicity tests only small numbers of animals were used. All animals survived five daily doses of 20 per cent of the previously determined LD_{50}, and all but one animal survived 40 per cent of the LD_{50} dose for five consecutive days. With six of the nine compounds tested, however, most of the animals failed to survive five daily doses of 80 per cent of the LD_{50} dose (see Table I).

SUMMARY AND CONCLUSIONS

1. Eight resorcinol derivatives have been compared with undecylenic acid for their fungistatic activity against *C. albicans* and *M. Gypseum*. The three disubstituted resorcinol derivatives, resorcinol diacetic acid, dibenzoate, and dimethyl ether were completely inactive against both organisms, while the diacetate was slightly active.

2. The three monosubstituted derivatives, monoacetate, monobenzoate, and monomethyl ether, and the ring substituted 4-chloro- derivative were all at least as fungistatic as undecylenic acid against both organisms. These results confirm the supposition that at least one free hydroxyl group must be present to achieve fungistatic activity.

The monomethyl ether and 4-chloro- derivative, although fungistatic, probably produce too much local irritation for effective use as fungistatic agents.

Resorcinol monobenzoate was fungistatic against both test organisms even in 0.1% and 0.01% concentrations, does not produce conjunctival irritation, and is approximately one half as toxic as the clinically used resorcinol monoacetate. Resorcinol monobenzoate appears worthy of further investigation as an antifungal agent.

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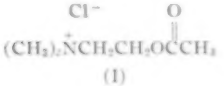
Isonitrosoketones*

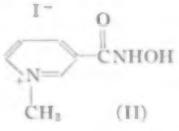
By GEORGE M. STEINBERG and JAMES BOLGER†

Several tertiary dialkylammonium and quaternary trialkylammonium isonitrosoketones have been prepared. These compounds were ineffective as reactivators of Sarin-inactivated acetylcholinesterase. The effect of the amine function on pK_a of the oximino group has been examined and found to be similar both in nature and magnitude to that found in aminocarboxylic acids.

THE ENZYME acetylcholinesterase which catalyses the hydrolysis of acetylcholine (I) is "irreversibly" inactivated by certain organophosphorus compounds, including diisopropyl phosphorofluoridate (DFP) and isopropyl methylphosphonofluoridate (Sarin), inactivation being caused by direct phosphorylation of the enzyme (1). It has been suggested by Wilson (2) that the activity of the enzyme resides in a small area which contains two spatially oriented sites: an anionic site which binds the positively charged nitrogen structure of the acetylcholine molecule (I) and an esteratic (basic) group which is properly oriented to attack the ester linkage. Since it is believed that in inactivation by organophosphorus compounds it is the esteratic site which is phosphorylated, Wilson (3) hypothesized that a good reactivating compound should combine a powerful nucleophile with a properly spaced quaternary ammonium group. In confirmation, he found



(I)



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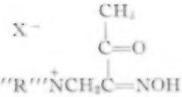

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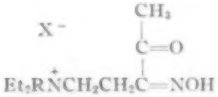

(IV)

that nicotinhydroxamic acid methiodide (II) is a particularly effective reactivator of diisopropyl phosphorylated enzyme.

The latter hypothesis is an attractive one although on the basis of more recent data (4–6), it appears to be somewhat of an oversimplification. We had found earlier (7) that acidic oximes (pK_a in the region 6–10.5) are powerful nucleophilic reagents towards Sarin and DFP and that one of them, 3-oximino-2-butanone, is rather effective *in vivo* as a prophylactic against poisoning by these agents (8). Hence we considered it of interest to combine the functions and prepared a series of nitrogen-containing isonitrosoketones of structures III and IV. These compounds were found to be neither particularly effective as enzyme reactivators (9) nor as *in vivo* prophylactics (10). It may be noted that on the basis of further study with oximes (see also reference 6) we now believe that the *aldoximino* group is required for enzyme reactivating activity.

Although the isonitrosoketones described in this paper were found to be of little value as antidotes or prophylactics for the phosphonofluoridates an examination of their acid-base properties has been made and is presented in this report. The efficacies of the active oximes are greatly influenced by pH and the rate of the reaction of oximes with Sarin and DFP is closely related to relative acidity; hence it was felt that the results of this study might contribute to our understanding of the nature of the chemotherapeutic process and so lead to the development of superior antidotes.

EXPERIMENTAL

The compounds listed in Table I were prepared from the corresponding bases by nitrosation with propyl nitrite under acidic conditions by the procedure of Bachman and Welton (11). In each case the free base could be obtained by ether extraction of an aqueous solution of the amine salt containing a 100% excess of potassium carbonate. To prepare the methiodides, the dried ether solutions were stripped and the residual oils (some crystallized) stored overnight at room temperature with an excess of methyl iodide. The solid products were isolated and purified by standard techniques.

The pK_a and neutral equivalent values were de-

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We wish to thank Dr. R. E. Plapinger and the members of the Chemotherapy Branch for helpful discussions and the Analytical Research Branch of the Research Directorate for having performed the analytical determinations.

TABLE I.—OXIMES, PHYSICAL AND ANALYTICAL DATA

Compound	Yield, Per Cent	M. p.,[a] °C.	Analysis					
			Calcd.			Found		
			N. E.[b]	C	H	N. E.	C	H
1-Dimethylamino-2-oximino-3-butanone hydrochloride	59	195	181	39.9	7.03	180	40.0	7.2
1-Dimethylamino-2-oximino-3-butanone methiodide	55	180	286	29.4	5.28	292	29.7	5.2
1-Diethylamino-2-oximino-3-butanone hydrochloride	75	150–3 (reported 154–5)[d]	209	...	...	206	...	...
1-Diethylamino-2-oximino-3-butanone hydrobromide	...	129–34	253	38.0	6.72	262	38.3	6.7
1-Diethylamino-2-oximino-3-butanone methiodide	35	149–51 156–58	312	34.4 N = 8.92; I = 40.40	6.05	308	34.6 N = 8.7; I = 40.2	6.0
1-(N-morpholino)-2-oximino-3-butanone hydrochloride	72.5[c]	221–4	222.5	43.2	6.8	223	43.5	7.0
1-(N-morpholino)-2-oximino-3-butanone methiodide	60	145	328	32.9	5.23	334	33.2	5.3
1-(N-piperidino)-2-oximino-3-butanone hydrochloride	55	228 (reported 228–9)[d]	220.5	...	...	220	...	...
1-(N-piperidino)-2-oximino-3-butanone methiodide	75	162	326	36.8	5.86	336	36.9	5.8
5-Diethylamino-3-oximino-2-pentanone hydrochloride	22	137	223	48.5	8.60	221	48.2	8.6
5-Diethylamino-3-oximino-2-pentanone methiodide	75	187–190	328	36.6	6.44	326	36.2	6.4

[a] The melting points of this group of compounds, although sharp, were subject to appreciable variation depending upon rate of heating. Thus, 1-diethylamino-2-oximino-3-butanone methiodide was observed to melt at 149–151° and at 156–158° in duplicate determinations. Samples were recrystallized from alcohol-ether or alcohol-acetone solution.
[b] N. E. = Neutral equivalent.
[c] Conversion. More than half of the unreacted morpholino ketone was recovered.
[d] Bachman and Welton (11).

termined from potentiometric titration curves. Approximately 100 mg. of sample was dissolved in 5 ml. of water (or 0.1 *N* hydrochloric acid if the substance was alkaline) and titrated with 0.1 *N* NaOH using a model G Beckman pH meter. pK_a values were taken as pH of half-neutralization, with corrections for sodium error and hydrolysis (12) when necessary. Under the conditions employed, hydrolysis was minor even at the highest pK_a value observed. With the most weakly acidic compounds, the second inflection point did not appear. However, the midpoint of the second ionization step could be calculated from the titration values of the first step. It is estimated that the pK_a values below 10 are accurate to *ca.* ± 0.1 unit. In general those above 10 are subject to greater error and are probably somewhat less accurate.

Absorption Measurements.—*Ca.* 100 mg. of accurately weighed sample was dissolved in 10 ml. of distilled water. A 2-ml. aliquot was admixed with the required volume of standard 0.1 *N* sodium hydroxide solution and diluted with distilled water to 10 ml. Light transmission through the yellow solutions was determined with a Klett-Summerson photoelectric colorimeter equipped with a #42 filter. Absorbance was observed to be linearly related to the percentage ionization of the oximino group in 1-diethylamino-2-oximino-3-butanone hydrochloride, 1-(N-morpholino)-2-oximino-3-butanone hydrochloride, 1-(N-piperidino)-2-oximino-3-butanone

hydrochloride and 1-(N-morpholino)-2-oximino-3-butanone methiodide.

The molar absorbance, A_{mol}, was calculated from the following equation (13).

$$A_{mol} = \text{Klett reading} \times 2/1000 \times \text{molarity}$$

DISCUSSION

The isonitrosoketones which were prepared for this study are included in Tables I and II. These compounds are amino acids or their quaternary salts which have an acid function that is considerably weaker than the carboxylic group of conventional amino acids, so that it was of interest to investigate the effect of the nitrogen grouping on the acidity of the oximes and to compare the results with similar observations which have been made with the amino carboxylic acids. The effect of the nearby protonated amino group or quaternary ammonium group is acid strengthening just as it is in the case of the conventional amino acids and is undoubtedly due to the powerful inductive and polar effects of the positively charged ammonium ion. The increase in acidity of the ketoximino group is similar in magnitude to that observed with the more strongly acidic carboxylic acid group (14). Thus, with the ammonium group in the α- position the pK_a is decreased by *ca.* 2.5 units and in the β- position by *ca.* 1 unit.

In acid solution the isonitrosoketones are colorless, while in alkali they are yellow. The color is associated with the resonating anion A, and its intensity is proportional to the degree of ionization of the isonitroso group.

(A)

Isonitrosoketones containing strongly basic functions would be expected to lose the first proton from the oximino group upon addition of alkali. In those cases in which the basic function in the position *alpha* to the oximino group is diethylamino, dimethylamino, or piperidino, it can be seen in Table II that the pK_1 values, corresponding to formation of the dipolar ion are substantially identical and are also equal to the pK_a values of the corresponding quaternary compounds. Where the strongly basic group is in the *beta* position the protonated ammonium group confers greater acidity on the oximino function than does the corresponding quaternary ammonium group.

Several of these compounds were examined colorimetrically (Table II). The observed relationship between pH and absorption characteristics of several of the compounds gives support to the conclusions derived from ionization data. In each case, the ionization step with pK_a value of close to 7 results in a deprotonated species with a molar absorbance greater than 20. In the case of the weakly basic *alpha* morpholino compound, the first ionization step involves loss of a proton from the nitrogen atom. Thus the pK_a values differ very little from those of the corresponding "parent" compounds and it is the second ionization step which gives a species having a molar absorbance greater than 20.

In the case of the strongly basic compounds, it is noteworthy that the acid-strengthening effect on the oximino group by the protonated and quaternized ammonium groups is identical in the *alpha* position while different when these groups are in the *beta* position. It may be of interest to speculate on the reason for this difference. With monodeprotonated compounds of this type, stabilization of the anion may result from (*a*) inductive effects along the molecular chain and (*b*) intramolecular ionic or hydrogen bonding which result in cyclic structures.[1] Solvent orientation would reinforce

Table II.—Ionization and Light Absorption Data of Oximes

R_1	R_2	R_3	pK_1	pK_2	A_{mol}[a] of Deprotonated Species: Mono	Di
(1) $R_1R_2R_3\overset{+}{N}CH_2C(=NOH)COCH_3\ X^-$						
(*a*) Me	Me	H	7.05	...[b]	...[b]	...[c]
(*b*) Me	Me	Me	7.05	...	...[b]	...
(*c*) Et	Et	H	7.05	11.4[c]	27.4	0
(*d*) Et	Et	Me	6.95	...	25.4	...
(*e*) (N-Morpholino)		H	6.1	9.15	3.3	25.9
(*f*) (N-Morpholino)		Me	6.9	...	43.6	...
(*g*) (N-Piperidino)		H	7.05	10.7	27.0	2.3
(*h*) (N-Piperidino)		Me	7.15	...	...[b]	...
(2) $Et_2R\overset{+}{N}CH_2CH_2C(=NOH)COCH_3\ X^-$						
(*a*) H			8.0	10.9	...[c]	...[b]
(*b*) Me			8.6	...	...[b]	...
(3) 3-Oximino-2-butanone			9.45	...	13.2	...

[a] Molar absorbance. For details see text. [b] Not determined. [c] Corrected for hydrolysis.

[1] This hypothesis requires that the oximino group be oriented *anti* to the adjacent carbonyl group. We believe this to be the case with the compounds studied here. Thus, Bachman and Welton (11) showed that compounds 1c and 1g (Table II) undergo the Beckmann rearrangement to yield the products corresponding to *anti* orientation. Also, all of the compounds reported in Table I give deep blue water-soluble chelates (unstable) with ferrous ammonium sulfate-pyridine reagent, which is characteristic of *anti* isonitrosoketones (Sidgwick, N. U., Taylor, T. W. J., and Baker, W., "The Organic Chemistry of Nitrogen," Oxford University Press, 1937, p. 195.)

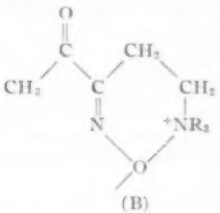
TABLE III.—pK_a VALUES OF AMINES

Compound	pK_a
1-Diethylamino-4-pentanone	10.1
Diethylamine (triethylamine)	11.1[a] (10.8)[a]
1-Diethylamino-3-butanone	9.25
1-Dimethylamino-3-butanone	8.95
Dimethylamine	10.7[a]
1-(N-morpholino)-3-butanone	6.6
Morpholine	5.6[b]

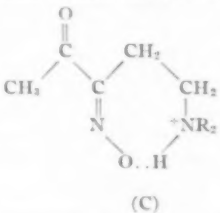
[a] Hodgman, C. D., "Handbook of Chemistry and Physics," 34 ed., Chemical Rubber Publishing Co., Cleveland, 1952, p. 1560.

[b] Ingram, A. K., and Luder, W. F., *J. Am. Chem. Soc.*, **64**, 3043(1942).

effects included in both (*a*) and (*b*) and therefore will not be considered. In the case of the *alpha* ammonium compounds, the inductive effects would appear to be overwhelming. With the *beta* ammonium compounds, the inductive effects diminish and anion stabilization due to ring formation becomes important. To explain the greater effect on pK_a of the protonated form in the latter compounds, one may assume that an ionic bonded ring (B) is



(B)



(C)

formed. In this case the degree of stabilization should be directly related to the closeness of approach of the oppositely charged groups. The protonated form would not only present less steric inhibition to close approach than the corresponding quaternary compound but may aid by the formation of a hydrogen bond (C).

The pK_a values of several of the intermediate aminoketones were determined and are compared with those of simple amines in Table III.

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(9) Reactivation studies were performed by members of the Enzyme Chemistry Branch under the supervision of Dr. B. J. Jandorf. They will be reported in detail elsewhere.

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Determination of Protoveratrine II.*

Separation of Protoveratrines A and B from Associated Alkaloids

By JOSEPH LEVINE and HENRY FISCHBACH

Commercial preparations of protoveratrines A and B were found to contain several associated veratrum alkaloids. The individual alkaloids may be separated by partition chromatography, using pH 3.0 buffer adsorbed on Celite® as stationary phase.

IN A RECENT PUBLICATION on the paper chromatography of several veratrum alkaloids (1), it was noted that protoveratrine A and germitrine have identical R_f values when developed by the systems described, while that of neogermitrine is very close to these. Since that time, a solvent system has been devised which affords a very marked separation of these alkaloids. In this system, however, the R_f values of germitrine, neogermitrine, and germitetrine (2) are identical. By means of a separate solvent system, the latter may be differentiated.

In the separation of several commercial samples of protoveratrines A and B into its components

* Received September 12, 1956, from the Division of Pharmaceutical Chemistry, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.

[1] An authentic sample of germitetrine was kindly furnished by the Research Laboratories of Ayerst, McKenna, and Harrison, Ltd.

by the procedure recently described (3), it was found that instead of the sharp demarcation which should be obtained between the pure individual alkaloids, a gradual elution of alkaloids continued after the passage of the amount of solvent which has been shown to be adequate for the complete elution of protoveratrine A. Examination of the eluted alkaloid by paper chromatography showed that the initial portions of solvent removed protoveratrine A; subsequent portions of eluate contained mixtures containing germitetrine, germerine, protoveratrine B, and another unidentified alkaloid more hydrophilic than protoveratrine B.

By appropriate modification of the partition system, as described in the experimental section, it was found possible to separate both protoveratrine A and protoveratrine B from the extraneous alkaloids. The appearance of an individual alkaloid in the eluate follows closely the final elution of the preceding alkaloid in several instances; the broad leeway which exists in the separation of pure protoveratrine A from protoveratrine B is thus lost in separating the more complex mixtures.

The procedure as described was successful in the case of those samples examined. It is not improbable that slight modification of the procedure, e. g., changing the volumes of solvent in the individual fractions, will be necessary in the separation of other mixtures. However, by the use of the paper chromatograms in pilot runs to monitor the separations, it is believed the procedure can be readily adapted to these cases.

EXPERIMENTAL PROCEDURE

Paper Chromatography.—Sharp separation of germitetrine, protoveratrine A, and protoveratrine B is achieved with the solvent system: 15 ml. ethylene chloride, 10 ml. Cellosolve acetate, and 1 ml. pyridine. The R_f values of the individual alkaloids are 0.5, 0.7, and 0.25, respectively. The identity of germitetrine[1] was established using the system: 20 ml. isooctane, 10 ml. methyl isobutyl ketone, and 2 ml. pyridine.

The chromatograms must be developed on paper distinctly moist with pH 3.5 buffer, as described previously (1). Little or no movement of alkaloid occurs on dry paper. Minor variations of R_f values result with variations in the moisture content of the paper; hence a known alkaloid is applied to each paper to serve as an internal standard. The location of the alkaloids on the developed chromatograms is detected with bromophenol blue, as before.

Chromatographic Separation of Alkaloids.—Suspend 2 grams of acid-washed Celite (4) in *ca.* 50 ml. benzene. While stirring vigorously (conveniently with a magnetic stirrer) add dropwise 2 ml. of a mixture of 3 parts (by volume) of McIlvaine's pH 3.0 buffer (5) and 1 part ethylene glycol. Transfer to a chromatographic column 18 × 280 mm. After the benzene drains off, tamp moderately firmly, in order to have a resultant flow rate of approximately 4 to 5 ml. per minute with the column full of solvent.

Pass a solution of 2 to 5 mg. of the alkaloid mixture dissolved in benzene or carbon tetrachloride over the column. (If the alkaloid is crystalline, solution in benzene is difficult. In this case, dissolve in 1 ml. of ethylene chloride, then add 3 ml. carbon tetrachloride).

Elute in the following sequence: (*a*) 150 ml. 3 parts (by volume) carbon tetrachloride + 2 parts ethylene chloride. (*b*) 100 ml. ethylene chloride. (*c*) 150 ml. 2 parts chloroform + 1 part ethylene chloride. (*d*) 100 ml. chloroform.

The first fraction will elute protoveratrine A quantitatively; the second will remove alkaloids which are more hydrophilic than protoveratrine A and less than protoveratrine B. Protoveratrine B is eluted in the third fraction. The final chloroform eluate will contain alkaloid more hydrophilic than protoveratrine B.

The separated alkaloids may be determined as previously described (3).

SUMMARY

A number of commercial preparations of protoveratrine A and B contain several other veratrum alkaloids together with the designated ones. By means of partition chromatography over Celite on which is incorporated a mixture of pH 3.0 buffer and ethylene glycol, the individual alkaloids are eluted consecutively with solvents of increasing solvent strength.

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Extraction of Morphine from Poppy Capsules and its Recovery by Ion Exchange*

By C. L. MEHLTRETTER and F. B. WEAKLEY

An efficient method for obtaining morphine from domestically grown poppies has been developed. Morphine was completely extracted from dried poppy capsules with water-saturated isobutanol containing 0.23% ammonia. By passage of the extract through a cation exchange resin bed, practically all of the alkaloid was adsorbed. Quantitative elution of morphine from the bed was achieved with dilute aqueous alkali. Crude morphine obtained by precipitation from the neutralized and concentrated eluate could be converted to pharmaceutical grade hydrochloride without difficulty. The overall recovery of morphine was 90%.

A STUDY of the recovery of the therapeutically important alkaloid morphine from mechanically harvested dried poppy capsules was undertaken in order to develop a process for production of this important alkaloid from a domestic source of raw materials. Investigation of the chemical and patent literature revealed a number of processes for the production of morphine from poppy straw (4, 5, 7, 10, 13). None, however, utilized ion exchange for the isolation of morphine. In recent years ion-exchange techniques have been introduced for the recovery of alkaloids from plant extracts (1, 2, 3, 6, 9, 11) and it is surprising that such procedures have not yet been applied to the opium alkaloids.[1] This fact and the relatively new developments in the synthesis of ion-exchange resins containing few cross-links for the preferential adsorption of large organic molecules have encouraged the study of ion exchange for the recovery of morphine from poppy capsule extracts.

The initial experimental survey of solvents for the extraction of morphine from dried poppy capsules showed water-saturated isobutanol to be very effective for this purpose. It was found that all of the morphine in the capsules was removed in two hours at 50°–60° with water-saturated isobutanol containing a small amount of ammonia. The low solubles and cation content of the extract were conducive to efficient utilization of ion exchange for the separation of the basic alkaloids present.

This paper describes the extraction of morphine from dried poppy capsules with ammoniacal water-saturated isobutanol and the nearly quantitative recovery of the alkaloid from solution by means of ion exchange. Zeo-Karb H,[2] a sulfonated coal, and Duolite C-10,[3] a synthetic sulfonic acid resin, were employed in this study for the adsorption of morphine. The anion-exchange resins, Duolite A-6 and A-7,[3] were used for adsorption of acids from the isobutanol effluents of the cation-exchange resin column.

EXPERIMENTAL

Analytical Procedure.—The liquid-liquid extraction procedure of Matchett and Levine (8, 12) was used for determination of morphine in process materials. It is recognized that the method is not specific for morphine, and in the presence of contaminating alkaloids gives high results. The errors, however, are expected to be consistent throughout the process so that the quantitative aspects of adsorption, elution, and recovery of morphine may be accepted with little reserve.

The dry poppy capsules were extracted with hot water for several hours in a Soxhlet apparatus, and the extract used in the analysis for morphine. Suitable aliquots of isobutanol extract and exhaust liquors were acidified and evaporated to near dryness to remove isobutanol prior to analysis for the alkaloid.

Extraction.—In the following experiments 11 separate extractions of fresh dried poppy capsules were made with ammoniacal water-saturated isobutanol. All but the first utilized isobutanol from the preceding extraction after it had passed through ion-exchange beds for removal of alkaloids and acids and was pretreated with ammonia.

Each extraction was carried out with 1 Kg. of ground (20-mesh) poppy capsules containing 9.2% moisture and 5.50 Gm. of anhydrous morphine (dry basis). In the first extraction, the capsules were covered with 5,200 ml. of water-saturated isobutanol containing 10 Gm. of ammonia. The mixture was digested at 50°–60° for 2 hours with occasional stirring. It was then filtered by suction and the residue agitated for 5 minutes with 3,500 ml. of fresh water-saturated isobutanol. After filtration, both liquors were combined to yield a total

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Mention in this article of firm names or commercial products does not constitute an endorsement of such firms or products by the U. S. Department of Agriculture.

[1] Applezweig (1) mentioned use of the ion-exchange technique in the isolation of morphine but failed to report further on this work.

[2] Manufactured by the Permutit Company, New York, N. Y.

[3] Products of the Chemical Process Company, Redwood City, Calif.

volume of 6,200 ml. Aliquots were removed for analysis for morphine, solubles, and ash (as sulfate). Water absorbed by the capsules was replaced by addition to the extract of 341 ml. of this solvent. After the alkaloids and acids were removed from the extract by ion exchange, 5,200 ml. of the liquor was conditioned for the following extraction by treatment with 10 Gm. of ammonia.

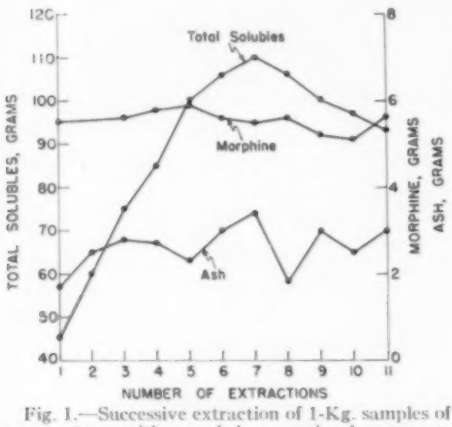

Fig. 1.—Successive extraction of 1-Kg. samples of poppy straw with recycled ammoniacal water-saturated isobutanol for 2 hours at 50–60°.

Figure 1 shows the morphine, solubles, and sulfated ash content of the extracts obtained from successive 1-Kg. samples of dried capsules by recycling the solvent. It is seen that the amount of morphine in each extract was fairly constant and was not appreciably affected by the solubles in the solvent. The solubles which accumulated in the solvent during the first seven extractions reached a maximum of 111 Gm. At this point the Duolite A-6 anion exchange resin was replaced by an equal weight of Duolite A-7 resin, whose higher capacity for removing color and solubles is shown by the decrease in solubles content of the solvent in extractions 8–11. It is also quite likely that an equilibrium had been established between the solubles in the extract liquor and the poppy capsules after the seventh extraction. The ash values of the extracts varied between 1.75 and 3.40 Gm./Kg. of dry capsules (Fig. 1). After passage through the cation- and anion-exchange resin beds, however, the values were reduced to the limits 0 to 0.45 Gm./Kg. of capsules (Table I). An unusually high figure of 0.85 Gm. was obtained only in the resin bed effluent of the first extraction.

Within experimental error of the analytical method, the recycled isobutanol removed all of the morphine from the capsules in each extraction.

TABLE I.—ZEO-KARB H ADSORPTION OF MORPHINE FROM ISOBUTANOL EXTRACTS OF 1 KILOGRAM OF POPPY CAPSULES[a]

Extraction No.	Morphine: Influent, Gm.	Effluent, Gm.	Adsorbed, Gm.	Adsorbed, %	Ash[c] in Effluent, Gm.
1	5.3	0	5.3	100	0.85
2	[b]	0	...	...	0
3	5.4	0	5.4	100	0.45
4	5.6	0	5.6	100	0.04
5	5.7	0.2	5.5	96	0
6	5.4	0.5	4.9	91	0
7	5.3	0.4	4.9	93	0.19
8	5.4	0.4	5.0	93	0.27
9	5.2	0.2	5.0	96	0.13
10	5.1	0.4	4.7	92	0
11	5.4	0.6	4.8	89	0.42

[a] Ammoniacal, water-saturated isobutanol effluent was used in all but the first extraction.
[b] A single analysis for morphine gave 4.1 Gm. which appears to be low.
[c] Ash determined as sulfate.

Ion Exchange.—Fundamental data were required since no information was available on the ion-exchange adsorption of morphine from organic solvents. For this purpose the sulfonated coal cation-exchange resin, Zeo-Karb H, was investigated in some detail. Later experiments were carried out with Duolite C-10, a synthetic sulfonic acid resin containing few cross-links.

Zeo-Karb H has a history of successful use for the adsorption of such alkaloids as nicotine (6, 11), quinine (2, 9), scopolamine, and atropine (1) from aqueous solution. Chief advantages to its use are (*a*) its porous nature, which allows the large alkaloid molecules to be readily adsorbed, (*b*) the ease with which the alkaloids can be displaced from it for recovery, and (*c*) its low cost.

The isobutanol effluent of the Zeo-Karb H necessarily contained small quantities of organic and inorganic acids liberated when the cations from dissolved salts were adsorbed. These were removed by an anion exchange bed of Duolite A-6 resin placed behind the Zeo-Karb H bed. If not adsorbed, such acids would be recycled with the isobutanol and would consume ammonia during conditioning of the solvent prior to extraction of the capsules. Ammonium salts thus formed, no doubt, would be solubilized to some extent by the solvent. Their passage through the cation-exchange bed would decrease its capacity for morphine.

It was necessary first to determine the adsorption-elution characteristics of Zeo-Karb H resin toward a water-saturated isobutanol solution of pure morphine similar in morphine content to the ammoniated isobutanol extract of poppy capsules. The basic morphine in solution suggested that the Zeo-Karb H be in the acid or hydrogen cycle for its adsorption. A study of the adsorption of morphine in the isobutanol poppy capsule extract was next made. The extract contained ammonium ions, resinous matter, and appreciable quantities of alkaloids other than morphine which are known to be present in poppy capsules. Thus it was expected that considerably more cation-exchange resin would be required to adsorb the morphine in the isobutanol extract than in the solution prepared with pure morphine.

In a preliminary run with 10.6 Gm. of Zeo-Karb H resin in a 1.0-cm. diameter column, 2.50 Gm. of morphine was adsorbed from isobutanol solution before breakthrough of the alkaloid was observed. Under similar conditions only 0.40 Gm. of morphine was adsorbed from the ammoniated isobutanol extract. Practically all the morphine was eluted from the resin bed with 35 ml. of 5% ammonia-methanol followed by 160 ml. of hot methanol. Because of the cost of recovery and the inconvenience

of using ammoniacal methanol, aqueous alkali was investigated as a morphine elution solvent in the following experiments.

A larger cation-exchange resin bed was prepared for morphine adsorption-elution studies on the isobutanol extracts obtained by recycling the deionized solvent. The bed was capable of adsorbing approximately 5 Gm. of morphine from the isobutanol extract of 1 Kg. of poppy capsules. It contained 200 Gm. of Zeo-Karb H (as received) and was 6.4 cm. in diameter. After backwashing and draining, the volume in the hydrogen cycle was 453 cu. cm. It was prepared for use by two successive treatments with 2% sodium hydroxide and sulfuric acid solutions. An increase in volume of 23% was noted when the bed was converted to the sodium cycle. No change in volume of the bed in the hydrogen cycle was observed after adsorption of alkaloids from the isobutanol extract.

The anion-exchange bed was 2.2 cm. in diameter and contained 30 Gm. of Duolite A-6 resin for the first seven runs. It was then replaced by an equal weight of Duolite A-7 resin. These resins were conditioned by treatment with 2% sulfuric acid followed by 2% sodium hydroxide solution.

Six to seven liters of water-saturated isobutanol extract containing 5.1–5.7 Gm. of morphine was passed continuously through the Zeo-Karb H and Duolite A-6 beds in series at 25 ml. per minute. The morphine was removed from the cation-exchange resin bed with alkali. Three hundred milliliters of 4% sodium hydroxide solution was introduced to the bed by upflow and allowed to remain for 15 minutes before draining. The process was repeated with fresh alkali. Two 300-ml. rinses of the bed with water followed. The alkali eluates and rinses were immediately acidified and combined for analysis for morphine. Since erratic displacement of morphine from the larger bed was obtained by this alkali elution procedure, efforts were made to improve the technique. Further investigation showed that reliable quantitative displacement of morphine could be realized by vigorously agitating the cation-exchange resin bed covered with 4% aqueous sodium hydroxide solution by aerating for 10 minutes before draining. No appreciable amount of morphine was lost by alkaline oxidation under these conditions probably because of the presence of natural antioxidants.

In all adsorption runs the Zeo-Karb H bed was regenerated by passing 1,200 ml. of 2% sulfuric acid through it at 27 ml. per minute. It was then rinsed with approximately 700 ml. of water. The anion-exchange resin was regenerated by downflow with 80 ml. of 2% sodium hydroxide followed by 450 ml. of water. The combined eluate and rinse

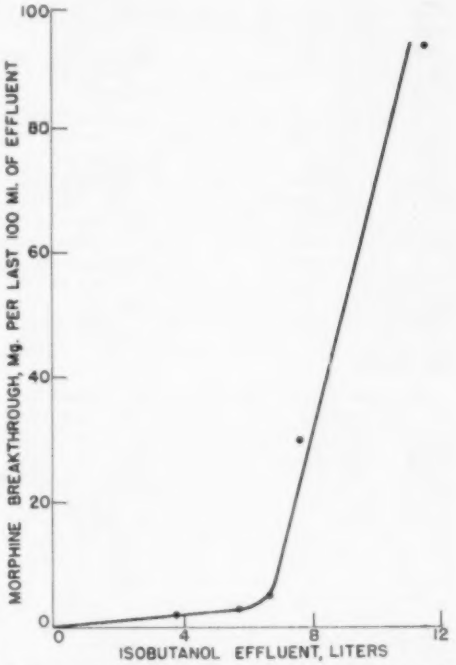

Fig. 2.—Relationship of morphine breakthrough and isobutanol effluent volume of Duolite C-10 bed.

of the regeneration procedure contained an average of 0.02 Gm. of morphine for all runs.

Table I shows the capacity of Zeo-Karb H resin for morphine in recycled isobutanol extracts of poppy capsules at low breakthrough of the alkaloid. A gradual loss in capacity with increased use of the Zeo-Karb H bed is apparent and is probably due to the build-up of resinous matter in the bed. Subsequent experiments showed, however, that this loss can be nearly eliminated by washing the resin bed in the acid cycle with isopropanol to remove adhering resinous matter.

Of interest also was the performance of Duolite C-10, a completely synthetic commercial sulfonic acid resin of low cross-linkage. Several stock solutions of isobutanol extract that were used in this study contained 1.5 to 1.8 Gm. of morphine per liter.

The bed of Duolite C-10 resin in the hydrogen cycle had a volume of 1444 cu. cm. Its diameter was 4.9 cm. and depth 76 cm. The resin as received was backwashed to remove fines, exhausted with

TABLE II.—DUOLITE C-10 ADSORPTION OF MORPHINE FROM ISOBUTANOL EXTRACTS OF POPPY CAPSULES

Adsorption No.	Influent, Gm.	Effluent, Gm.	Adsorbed, Gm.	Adsorbed, %	Eluted, Gm.	Eluted, %
1	11.29	0.55	10.74	95	10.51	98
2	10.83	0.55	10.28	95	10.07	98
3	10.99	0.82	10.17	93	10.43	103
4	10.63	0.39	10.24	96	10.06	98
5	10.61	0.31	10.30	97	10.81	105
6	11.66	0.64	11.02	95	10.43	95
7	11.36	0.61	10.75	95	10.45	97

4% sodium hydroxide, and regenerated with 5% sulfuric acid three times before use. The original B and D depth of 76 cm. was unchanged after all adsorption runs had been made.

Several preliminary experiments were carried out to determine the volume of isobutanol extract to use for the adsorption of morphine by the resin bed at low breakthrough levels. The relationship of volume of isobutanol effluent to morphine breakthrough on the Duolite C-10 bed is shown in Fig. 2. An effluent volume of 6600 ml. appeared to be satisfactory for a practical separation of morphine.

In seven consecutive runs which were made next, isobutanol effluent volumes of 6400 to 6800 ml. (influent 6600 to 7000 ml.) were collected from the resin bed to obtain morphine adsorption and elution data (Table II). The effluents were not recycled in these trials.

Morphine Adsorption and Elution Procedure.—A volume of 6,884 ml. of water-saturated isobutanol extract (pH 8.3) was passed down through a Duolite C-10 cation-exchange resin bed which had been used previously for morphine adsorption, displacing the water that had covered the bed. A flow of 120 ml. per minute was maintained under constant pressure. The initial water effluent was separated; the volume of isobutanol effluent was 6,770 ml. after the bed was drained for 30 minutes. The solvent holdup of the bed was, therefore, 184 ml. By vacuum, one liter of 4% aqueous sodium hydroxide solution (4 Gm./100 ml.) was introduced to the bed by upflow and aerated vigorously for 10 minutes. The bed was then drained at 120 ml. per minute while it was rinsed with distilled water by downflow. Two liters of water was required to remove practically all morphine from the bed. The total alkali eluate was immediately neutralized to pH 6.9 with approximately 15 ml. of concentrated sulfuric acid. Resinous matter separated during the neutralization and coated the sides and bottom of the vessel. The brown eluate was removed from most of the resinous material by careful decantation, and then was vacuum-filtered through a cotton plug to remove a small amount of suspended solid material. The filtrate was collected. (Later it was added to analogous liquors from succeeding runs, concentrated, and worked up for recovery of crystalline crude morphine.)

Immediately after the alkali elution step, the resin bed was regenerated to the hydrogen cycle by passing 1 liter of 5% (5 Gm./100 ml.) sulfuric acid up through the bed by vacuum. The bed was aerated for 10 minutes and drained at 110 ml. per minute while it was rinsed with distilled water. About 5700 ml. of water was required to reach an effluent pH of 3.3 to 3.8 with the bed just covered with water. The bed was then ready for the next adsorption.

Isolation of Crude Crystalline Morphine.—The neutralized alkali eluates of the first four adsorption runs (Table II) were combined and filtered by vacuum through a cotton plug. The approximately 12 liters of filtrate of pH 6.9 containing 38.5 Gm. of morphine (93% recovery) was evaporated *in vacuo* in a rising film circulating still to a volume of 800 ml. The liquor was drained from the still and the latter rinsed with 3000 ml. of water. The combined liquor and rinse was then treated with 25% ammonium hydroxide to precipitate morphine at pH 9.1. After standing overnight at room temperature, the crystalline morphine was filtered by suction and washed with water several times. After it was dried at 60° for 6 hours it weighed 48.6 Gm. The light-brown product containing 65% morphine (82% recovery) and 5% moisture could readily be converted to pharmaceutical grade morphine hydrochloride. Morphine remaining in the mother liquor could be recovered by ion exchange or returned to the process by addition to a subsequent concentrate so that a total recovery of morphine of close to 90% of theory was achieved.

CONCLUSIONS

Zeo-Karb H and Duolite C-10 which are sulfonic acid type cation exchange resins containing few cross-links, can effectively adsorb morphine from water-saturated isobutanol extracts of dried poppy capsules. A strict comparison of the performance of these resins could not be made because different procedures were used in each case. It was shown, however, that Zeo-Karb H had 55% greater adsorptive capacity for morphine than Duolite C-10. The morphine adsorbed can readily be eluted quantitatively from the resins with dilute aqueous alkali. Maintenance by such resin beds of alkaloid capacity after repeated adsorptions and elutions indicates the practicability of ion exchange separation for large-scale recovery of morphine from dry poppy capsules.

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The Statistical Evaluation of a Biological Assay for Antiemetic Drugs*

By JOSEPH L. CIMINERA, CLEMENT A. STONE, and JOHANNES IPSEN†

The use of Ipsen's method for obtaining optimum appropriate scores in the evaluation of a biological assay for antiemetic drugs is discussed. The method combines graded observations for quantal responses into one continuous score system. Evaluation based on a quantal response was shown to be only two-thirds as efficient as that based on a quantitative response using optimum scores.

IN MANY QUANTAL response assays additional information of a quantitative nature is available for individual responses. For example, in assays involving deaths and survivors, records may be available that show the time to death and survival with or without symptoms of varying severity. Attempts have been made to combine graded observations on responders and nonresponders into one continuous score system. The assignment of a score system by graphical trial and error methods to assure a normal distribution of responses and linearity of dose response curves is too subjective and does not guarantee maximum efficiency in the utilization of biological data. Ipsen (1) recently published a procedure for assigning appropriate scores that are optimum in the sense that the variance of the linear regression of the mean scores on log dose is the highest possible fraction of the total variance of a set of graded dose experiments.

We have applied Ipsen's method to a biological assay for antiemetic drugs. The responses in this assay were classified originally as the proportion of animals vomiting or not vomiting (quantal response). However, there were quantitative aspects to both types of response. In the dogs that vomited we were able to record the number of vomitings in a thirty minute period, and in the dogs that did not vomit it was possible at times to record signs of retching. However, the latter occurred too infrequently to be useful for quantification. We hoped to show that evaluation based on a quantitative response would give more information than evaluation based on a quantal response.

The purpose of this paper is to illustrate a useful application of Ipsen's method. Calculations are given in detail for a symmetrical 6-point assay. The application to single assays requires some modification of the procedures given in Ipsen's original paper (1) which is concerned mainly with a score system applicable to a large number of assays.

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EXPERIMENTAL

Twelve dogs were used in the assay. Over a period of time each dog received three graded dose levels of a standard and test preparation intravenously. Thirty minutes after administration of the drug, each dog was given 0.05 mg./Kg. apomorphine hydrochloride subcutaneously. The dogs were observed for thirty minutes and the number of times each dog vomited was recorded.

The three dose levels for standard and test preparation were $S_1 = 0.125$, $S_2 = 0.25$, $S_3 = 0.5$ mg./Kg. and $T_1 = 0.25$, $T_2 = 0.5$, $T_3 = 1.0$ mg./Kg., respectively. The order of dosage was varied in each dog according to two Latin Square designs as shown in Table I. These designs were intended to segregate variations due to dogs and days. A one day rest period was used between test sessions with each dog to eliminate any possible drug carry over effects.

RESULTS AND CALCULATIONS

Table I shows the structure of the test procedure and gives the number of times each dog vomited during the thirty minute period following challenge with apomorphine hydrochloride.

Evaluation Based on a Quantal Response.—Table II shows the number of dogs not vomiting out of the total number tested on each dose of standard and test preparation.

The results were evaluated by probit analysis (2). There was no evidence of heterogeneity and the dose response curves were parallel within the limits of experimental error. The potency of the test preparation was estimated to be 35% of standard with 95% fiducial limits of 19% and 58%.

Estimation of Appropriate Scores.—Table III illustrates the procedure for estimating appropriate scores for each preparation.

For symmetrical assays it is convenient to code the doses using orthogonal coefficients (3). For a 6-point symmetrical assay, the coded doses are -1, 0, $+1$. Table III shows the number of dogs, a, in each vomiting category for each dose of each preparation. The vomiting categories are arranged in sequence from the largest observed number of vomitings to the smallest (i. e., from 3 to 0). The last column of the table shows the total number of dogs tested at each dose level, $n = 12$. The subtotals, A, represent the total number of dogs in each vomit-

TABLE I.—NUMBER OF VOMITINGS IN A PERIOD OF THIRTY MINUTES FOLLOWING A SUBCUTANEOUS INJECTION OF APOMORPHINE HYDROCHLORIDE 0.05 MG./KG.

Square I

Day	Dog 1	2	3	4	5	6
1	S_2—1	T_1—1	T_2—0	T_6—1	S_2—0	S_1—1
3	T_1—3	S_5—0	T_3—0	S_1—1	T_2—1	S_2—1
5	T_2—2	T_4—0	S_3—0	S_2—1	S_1—0	T_1—2
7	S_2—1	T_3—1	S_1—0	T_1—3	S_3—0	T_3—0
9	S_1—3	S_4—1	T_1—0	T_2—1	T_3—0	S_3—0
11	T_3—1	S_1—2	S_3—0	S_3—1	T_1—1	T_2—1

Square II

Day	7	8	9	10	11	12
2	T_1—1	S_3—0	S_1—0	S_6—0	T_3—0	T_2—2
4	S_2—0	T_4—0	T_2—0	S_3—0	S_1—2	T_1—3
6	S_1—1	T_1—1	S_3—0	T_3—1	T_2—1	S_2—0
8	S_3—0	S_1—2	T_1—2	T_2—1	S_2—1	T_3—0
10	T_3—0	T_5—0	S_2—1	S_1—1	T_1—2	S_3—0
12	T_2—0	S_4—0	T_4—0	T_1—2	S_3—0	S_1—1

TABLE II.—RESULTS BASED ON A QUANTAL RESPONSE

Standard preparation	Test preparation
$S_1 = {}^3/_{12} = 25.0\%$	$T_1 = {}^1/_{12} = 8.3\%$
$S_2 = {}^6/_{12} = 50.0\%$	$T_2 = {}^4/_{12} = 33.3\%$
$S_3 = {}^{10}/_{12} = 83.3\%$	$T_3 = {}^9/_{12} = 75.0\%$

ing category. The subscripts, S and T, refer to standard and test preparation, respectively. The other computations are illustrated for vomiting category 1 of the standard preparation:

$$d_S = \Sigma xa = (-1)(5) + (0)(6) + (+1)(2) = -3$$

$$c_S = d_S/A_S = (-3)/13 = -0.2308$$

$$\text{Scores} = Q[(c_j - c_1)/(c_m - c_1)] = 4[(c_j + 1)/(0.3684 + 1)] = 4[(-0.2308 + 1)/(1.3684)] = 2.25$$

where

Q = any convenient quantity such as 100 or m, the number of categories.
c_j = c for the j'th category.
c_1 = c for the first category.
c_m = c for the last category.

TABLE III.—ESTIMATION OF APPROPRIATE SCORES

Coded dose x	Number of dogs, a, in vomiting category 3	2	1	0	Subtotals n
Standard Preparation					
−1	1	3	5	3	12
0			6	6	12
+1			2	10	12
Subtotals, A_S	1	3	13	19	$36 = N_S$
d_S	−1	−3	−3	7	
c_S	−1	−1	−0.2308	0.3684	
Scores	0	0	2.25	4	
Test Preparation					
−1	3	4	4	1	12
0		2	6	4	12
+1			3	9	12
Subtotals, A_T	3	6	13	14	$36 = N_T$
d_T	−3	−4	−1	8	
c_T	−1	−0.6667	−0.0769	0.05714	
Scores	0	0.85	2.35	4	

TABLE IV.—ESTIMATION OF A COMMON SCORE SYSTEM

Vomiting category	$A = A_S + A_T$	$d = d_S + d_T$	$c = d/A$	Common scores C
3	4	−4	−1	0
2	9	−7	−0.7778	0.61
1	26	−4	−0.1538	2.33
0	33	15	0.4545	4
Sums	$N = N_S + N_T = 72$	0[a]		

[a] As a check, the sum of the d's should always equal zero.

Using this score system, the first score will always be zero and the last score will always be Q.

Computation of appropriate scores is useful only if one can arrive at a set of scores that is appropriate for all assays of the same type. In particular, for a single assay one should be able to arrive at a set of scores that is appropriate for both the standard and test preparations, and the choice of such a set should cause no significant loss of information. A test for loss of information is given in the discussion. The common score system, C, is obtained in the same manner as the individual score systems. The computations are summarized in Table IV.

Evaluation Based on a Quantitative Response.—Substituting the common scores for the individual responses in Table I one obtains the mean scores shown in Table V.

TABLE V.—RESULTS BASED ON A QUANTITATIVE RESPONSE (MEAN SCORES)

Standard preparation	Test preparation
$S_1 = 2.12$	$T_1 = 1.31$
$S_2 = 3.16$	$T_2 = 2.60$
$S_3 = 3.72$	$T_3 = 3.58$

The results were evaluated using standard methods (3). The analysis of variance is shown in Table VI.

19% and 58% for the quantal response to 24% and 48% for the quantitative response.

DISCUSSION

Test for Loss of Information Due to Using a Common Score System.—A composite statistic, χ^2_c, is estimated for individual score systems with $(m - 1)$ degrees of freedom [i. e., 1 d. f. for linear regression and $(m - 2)$ d. f. for individual score systems] from:

$$\chi^2_c = [N_g \cdot \Sigma(d^2_c/A_g)]/[n \cdot \Sigma x^2] = k \cdot \Sigma(d^2_c/A_g)/\Sigma x^2$$

where

k = number of doses for each preparation
g = subscript denoting the g'th preparation

For the standard preparation:

$$\chi^2_S = k \cdot \Sigma(d^2_S/A_S)/\Sigma x^2 = 3(1/1 + 9/3 + 9/13 + 49/19)/[(-1)^2 + (0)^2 + (+1)^2] = 10.91$$

Similarly, for the test preparation, $\chi = 15.47$.

When a common score system is computed, we use the same degrees of freedom $(m - 2)$ as for an individual system. Theoretically, this loss of $(m - 2)$ degrees of freedom would have to be distributed some way over all the individual assays from which the common score system is derived. If the number of individual assays is large, individual tests for loss of information due to using a common score system will not be influenced by throwing away $(m - 2)$ degrees of freedom. However, where only a few assays are involved (in the present case only two), some allowance has to be made. In such cases no attempt is made to evaluate the loss in individual assays, but rather the loss in the total experiment. To do this we first compute a new chi square for each preparation using the common score system, C, in place of the individual systems from:

$$\chi^2_{\delta_g} = k(\Sigma d_g C)^2/(\Sigma x^2 \cdot [C^2]_g)$$

where

$$[C^2]_g = \Sigma A_g C^2 - (\Sigma A_g C)^2/N_g$$

TABLE VI.—ANALYSIS OF VARIANCE FOR QUANTITATIVE RESPONSE

Source of variation	Degrees of freedom	Sums of squares	Mean squares	F ratio
Squares	1	0.8558	0.8558	1.03
Rows (days) in squares	10	4.2375	0.4228	0.51
Columns (dogs) in squares	10	35.3489	3.5349	4.26[b]
Doses	5	51.4636		
Preparations	1	4.5854	4.5854	5.52[a]
Linear regression + score system[c]	3	44.8727	14.9576	18.01[b]
Lack of parallelism	1	1.3501	1.3501	1.63
Curvature	1	0.6228	0.6228	0.75
Opposed curvature	1	0.0327	0.0327	0.04
Residual error	43	35.7094	0.8305	
Total	71	127.6152		

[a] Significant ($P < 0.05$).
[b] Significant ($P < 0.001$).
[c] This is a composite statistic consisting both of the component for linear regression (1 *d. f.*) and the "costs" of evaluating the appropriate score system which maximalizes the linear regression. Among m categories one can always arbitrarily assign values to the first and last (in this case 0 and 4) and then compute the remaining scores in relation to the two fixed values. Hence, of the m categories, only $(m - 2)$ can vary and accordingly the system is determined with $(m - 2)$ degrees of freedom. The latter are taken from the residual error and added to the one degree of freedom for linear regression, making a total of $(m - 1)$ degrees of freedom.

There were no significant day to day differences, but variations among dogs were highly significant ($P < 0.001$). Reference to Table I reveals that some dogs were more sensitive to treatment than others. For example, dog 3 showed no evidence of vomiting at any dose of drugs tested, indicating a high sensitivity to both drugs (or resistance to challenge with apomorphine hydrochloride). There was no indication of curvature and the dose response curves were parallel within the limits of experimental error. The significant F ratio for preparations indicates that we misguessed the relative potency of the test preparation.

The estimate of relative potency was 35%, the same as that based on a quantal response. However, the 95% fiducial limits were narrowed from

For the standard preparation, we obtain

$$\begin{aligned}\Sigma d_S C &= (-1)(0) + (-3)(0.61) + (-3)(2.33) + (7)(4) \\ &= 19.18 \\ [C^2]_S &= \Sigma A_S C^2 - (\Sigma A_S C)^2/N_S \\ &= (1)(0)^2 + (3)(0.61)^2 + (13)(2.33)^2 + (19)(4)^2 - [(1)(0) + (3)(0.61) + (13)(2.33) + (19)(4)]^2/36 \\ &= 50.97 \\ \chi^2_{cs} &= k(\Sigma d_S C)^2/(\Sigma x^2 \cdot [C^2]_S) \\ &= 3(19.18)^2/[(2)(50.97)] = 10.83\end{aligned}$$

Similarly, for the test preparation we obtain $\chi^2_{cT} = 15.43$. The final computations are summarized in Table VII.

TABLE VII.—TEST FOR LOSS OF INFORMATION DUE TO USING A COMMON SCORE SYSTEM

Preparation	χ^2_c	*d. f.*	$\chi^2_{c_o}$	*d. f.*	
Standard	10.91	3	10.83	1	+2
Test	15.47	3	15.43	1	
Sums	26.38	6	26.26	4	

Loss of information = $\Sigma\chi^2_{c-c_o} = 26.38 - 26.26 = 0.12$; $d.f. = 6 - 4 = 2$

$P > 0.90$, indicating no significant loss of information due to using a common score system.

Efficiency of Using a Common Score System.—It may be of interest to express the loss due to using a common score system in terms of relative efficiency, E. This may be estimated from

$$E_o = [\chi_c^2 (N_o - \chi_c^2)]/[\chi_c^2 (N_o - \chi^2_{c_o})]$$

Thus, for the standard preparation we obtain

$$\begin{aligned}E_S &= [\chi^2_{cs} (N_S - \chi^2_S)]/[\chi^2_S (N_S - \chi^2_{cs})] \\ &= (10.83)(36 - 10.91)/(10.91)(36 - 10.83) \\ &= 0.990\end{aligned}$$

Similarly, for the test preparation we obtain $E_T = 0.995$, indicating that there was practically no loss in efficiency using the common score system in place of individual score systems.

Adequacy of a Single Assumed Score System.—Instead of using the optimum appropriate score system, we may want to test the adequacy of a simple assumed score system, $Z = 0, 1, 2, 3$. Applying the same computational procedures that were used for testing the adequacy of the common score system we estimate for each preparation

$$\chi^2_o = k(\Sigma d_o Z)^2/(\Sigma x^2 \cdot [Z^2]_o)$$

This gives $\chi^2_{zs} = 10.51$ for the standard preparation and $\chi^2_{zT} = 15.24$ for the test preparation.

When we "fix" all values, as in the case of an assumed score system, Z, the value χ^2_z is dependent only on the slope variation. This statistic will be determined, therefore, with one degree of freedom. The test for loss of information implies that we lose something by guessing all the scores instead of computing their optimal value. This loss is weighed by the difference in degrees of freedom.

For the standard and test preparations we obtain $\chi^2_{s-zs} = 10.91 - 10.51 = 0.40$ and $\chi^2_{T-zT} = 15.47 - 15.24 = 0.23$, respectively. Each difference is determined with $(m - 1) - 1 = m - 2 = 2$ degrees of freedom, so that in each case $P > 0.80$, indicating that in this assay there would have been no significant loss of information if we had adopted the simple assumed score system.

In terms of relative efficiency we obtain $E_{ZS} = (10.51)(36 - 10.91)/(10.91)(36 - 10.51) = 0.95$ and $E_{ZT} = (15.24)(36 - 15.47)/(15.47)(36 - 15.24) = 0.97$ for the standard and test preparations, respectively.

Efficiency of Quantal Response.—We showed in a previous section that the 95% fiducial limits were narrowed considerably by using a quantitative response in place of a quantal response. An elegant way of showing that the quantal response is less efficient than the quantitative response is to compute the efficiencies for a score system, $U = 0, 0, 0, 1$ relative to the common score system, C. Proceeding as in the last section, we obtain $\chi^2_{US} = 8.19$ and $\chi^2_{UT} = 11.21$, so that $E_{US} = (8.19)(36 - 10.83)/(10.83)(36 - 8.19) = 0.68$ and $E_{UT} = (11.21)(36 - 15.43)/(15.43)(36 - 11.21) = 0.60$. This shows a "saving" in one third of the animals.

SUMMARY

(1) The statistical evaluation of a biological assay for antiemetic drugs is discussed. The assay was evaluated on the basis of both a quantal response and a quantitative response.

(2) It was shown that the evaluation based on a quantal response was only two-thirds as efficient as that based on a quantitative response.

(3) A method due to Ipsen for obtaining optimal scores was discussed for a symmetrical assay.

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The Chemistry of Polyvinylpyrrolidone-Iodine*

By S. SIGGIA

Polyvinylpyrrolidone is a water-soluble, physiologically acceptable polymer which has the ability to tie up iodine. The PVP-iodine resulting from this combination retains the germicidal and chemical nature of the iodine yet it is more water-soluble than iodine alone; it provides a more stable form of iodine in solution than iodine alone or some of the more common iodine preparations such as iodine tincture and Lugol's solution. The iodine present in PVP-iodine is not as irritating to tissue and mucosa as other iodine preparations of the same strength, and the toxicity of the iodine is reduced.

IODINE has long been known for its germicidal action. It is reported (17) that iodine was used as early as 1819, the first report on the germicidal activity having been made in 1873 by Davaine. Gershenfeld and Miller (5) show the phenol coefficient of various iodine solutions to vary from 1.7 to 2.35 for each per cent of iodine present, or from 170 to 235 based on the total iodine present. They used the Food & Drug Administration method with *E. typhosa* and *Staph. aureus* as test organisms. Other work on the germicidal efficiency of iodine has been carried out by Salle, *et al.* (21, 22), Nye (19), and McCulloch and Fuller (18). Much work has also been reported on the germicidal effectiveness of iodine for water purification (14).

The efficiency of iodine as a skin disinfectant is indicated in reports by Simmons (17, 26) and Sinclair (17). Iodine also exhibits fungicidal properties (15, 17, 23). In fact, Emmons (4) reported a phenol coefficient of approximately 3000 using *Trichophyton gypsum* and *Monilia albicans*.

Iodine has also been known as a virucide, and was used for the treatment of the common cold and influenza (6, 9) as far back as 1919. Other viruses affected by iodine are poliomyelitis, vaccinia virus, herpes simplex, rabies, trachoma, tobacco mosaic virus, and others (6). Iodine has also been reported to be effective in the treatment of arthritis and rheumatism (8). In the field of veterinary medicine, iodine has been found to exert action against ringworm (10), lungworm disease (13), mastitis (12), and avian coccidiosis (11).

In view of all the possible uses of iodine, it would seem that iodine would be more widely used. However, there are shortcomings in the use of iodine. Free iodine, the form active against the organisms mentioned above, is not very soluble in water (0.335 Gm./L.) (24). Either a solvent such as ethanol has to be used as in iodine tincture, or potassium iodide as a solubilizing agent as in Lugol's solution. Another disadvantage of the use of iodine is the irritating nature of iodine itself or the solvents or salts used in formulations. Some iodine preparations are not stable because of loss of iodine. This may be due to its volatility or to its relatively high reactivity (usually oxidizing action) which may cause iodine to react with the solvent or with other organic components in formulations. The toxicity of free iodine (7) is another reason for the limited use of the material. Iodine is also a primary irritant and sensitizer to skin and mucosa.

CHEMICAL AND PHYSICAL CHARACTERISTICS

Polyvinylpyrrolidone (PVP) is a water-soluble polymer which is physiologically acceptable to animals and humans. It has been used in the United States and Europe as a blood plasma extender, drug vehicle, suspending agent (protective colloid), and tableting aid. This material, made by the polymerization of vinyl pyrrolidone, has the ability to combine with many materials, and thereby often markedly changing their physical and toxicological properties. Iodine is strongly "complexed" by PVP. This complexing action minimizes some of the difficulties previously experienced with the use of iodine. The PVP-iodine (PVP-I) "complex" is a powder and is quite water-soluble. These solutions and powders are quite stable to storage since the iodine vapor pressure is reduced essentially to zero, and the PVP-I "complex" is also quite chemically stable. The oral toxicity of iodine is also reduced by the PVP, probably due to the fact that the iodine is not present as such but tied up by the PVP. This permits the internal use of larger amounts of iodine without toxic effects. The incorporation of iodine with PVP also eliminates or at least lessens the irritation experienced in the use of other iodine preparations of comparable strength.

When iodine and PVP are mixed there is some chemical reaction between the two; the iodine oxidizes unsaturated end groups or residues in the PVP, resulting in an amount of iodide ion being formed. Apparently, this reaction is carried to completion in the preparation of the PVP-I so that

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no further loss of iodine occurs in the powder after the initial preparation. The finished product contains about two-thirds of the original amount of iodine still as iodine. This is titratable with thiosulfate and is termed *available iodine* since it is the effective form of iodine available for germicidal purposes. The iodide ion content of the final product accounts for practically all the remainder of the iodine; a very small percentage of iodine becomes organically linked and does not appear as available iodine or as iodide ion.

The bond between the PVP and the available iodine is not completely understood. The fact that there is a bond cannot be disputed because, among other things, the iodine cannot be extracted from either the solid PVP-I or the solutions using solvents such as carbon tetrachloride. The fact that the iodine vapor pressure is reduced essentially to zero and the fact that the iodine is solubilized in water to such a degree is convincing evidence that the iodine is linked in some way to the PVP. Yet this iodine is available for therapeutic use and also undergoes all the chemical reactions characteristic of iodine. It was thought that an equilibrium existed between iodine combined with PVP and free iodine, PVP.Iodine $\rightleftarrows$ Iodine + PVP, but if this is correct, then it should be possible to extract iodine continually with carbon tetrachloride from the PVP-I, but this is not the case. Most often no iodine at all is removed with the solvent.

TABLE I

	% of Total Available Iodine Lost After 20 Hours[a]	
	0.03%	1.0%
Iodine alone	97%	...
PVP-Iodine	10%	<3%[b]
Tincture of Iodine	10%	<3%[b]
Lugol's Iodine	66%	18%

[a] Stored at room temperature in open beakers with volume maintained constant. Iodine determined before and after storage using titration with thiosulfate.

[b] The volume in the beakers could not be controlled to better than ±3% so this became the limiting factor in the experiment.

Oster and Immergut (20) showed that PVP will alter the ultraviolet absorption spectrum of iodine. This indicates some interaction. They postulate that the iodine causes the PVP molecules to assume a helical form with the iodine atoms inside the helix. Eirich (3) states that one atom of iodine is held per monomer unit in the PVP and indicates that the bond between the iodine and PVP is ionic in character.

PVP will tie up iodine to the extent of approximately one part iodine to two or three parts PVP. If the iodine ratio is increased above this value, then the iodine vapor pressure rises very sharply and also iodine can then be extracted with carbon tetrachloride. PVP-I can be used in any proportion of iodine to PVP below the ratio just mentioned. The most common ratio used so far has been one containing 10 Gm. of available iodine per hundred Gm. of product. This is called PVP-I[10] and contains approximately 85% PVP, 10% available iodine, and 5% iodide ion formed in the process.

Iodine Solubilization by PVP.—It was stated above that PVP will solubilize iodine, thereby making possible aqueous solutions of higher iodine content. Iodine alone is soluble to the extent of 0.034% in water at 25° whereas it is soluble to the extent of 0.58% in a 1% aqueous PVP solution under the same conditions, a 17-fold increase caused by 1% PVP. It is possible to make aqueous solutions containing 10% of available iodine with the use of large amounts of PVP.

Stability of PVP-Iodine Powders and Solutions.—The instability of iodine preparations may be due either to the volatility or to the reactivity of iodine. Table I shows the stability of solutions of iodine in various formulations toward volatilization. The solutions were stored in open beakers to favor volatilization, but the volumes were kept constant by the addition of the particular solvent involved. The series at 0.03% concentration was made to compare with the saturated aqueous iodine. The 1% iodine solutions were prepared to compare the volatilization at a higher iodine level. To get all iodine formulations to a common iodine level they were diluted with their respective solvents; PVP-iodine and Lugol's were diluted with water, and iodine tincture was diluted with 1:1 ethanol and water.

To check the chemical stability of the various iodine preparations, solutions were made to contain 1% of iodine, the diluents again being water in all cases except the tincture, where the diluent was 1:1 ethanol-water. These solutions were then placed in glass-stoppered bottles, sealed with plastic tape, and stored at 42°. The results are shown in Table II. The saturated aqueous solution of plain iodine was not included in this series since most of the iodine sublimed out of the water and crystallized on the portion of the bottle above the liquid. The iodine was therefore not all in contact with the solution, and the results could not be considered representative of chemical stability. It can be presumed that no iodine is lost through chemical reaction in the case of just a saturated iodine solution since there is nothing present for the iodine to react with. Distilled water was used since tap water may contain a small amount of oxidizable impurities.

The data show that PVP-iodine solutions are at least as stable, if not slightly more stable, than Lugol's solution. Iodine tincture is poor in comparison, probably due to oxidation of the alcohol solvent by the iodine. The instability results on iodine tincture are substantiated by La Wall and Tice (16) who found that iodine was lost in solutions containing primary alcohols; ethanol was included in their experiments. They also postulated the mechanism to be oxidation of the alcohol. Beal, Waters, and Block (1) maintain that iodine tincture is stable when stored in clear, amber, or blue glass

TABLE II

	% of Total Available Iodine Lost on 32 Day Storage at 42°[a]
Tincture of Iodine	47
Lugol's Iodine	8
PVP-I (10)[b]	5
PVP-I (20)[b]	2

[a] 1% Available Iodine solutions were used. Iodine determined by thiosulfate titration.

[b] The number indicates the per cent available iodine in the powders.

stoppered bottles. Experiments carried out in our laboratory at room temperature, 42° and 65°, in clear and amber glass-stoppered bottles (sealed tubes were used for 65° storage to prevent evaporation) showed very distinct losses of iodine in all cases.

PVP-iodine powders have been stored in plain glass-stoppered bottles (no tape or seal) for three years at 65°. The various powders stored contained 2.5, 5.0, and 10.0% of available iodine. In all cases no detectable iodine (<0.05% absolute) was lost from any of these in spite of the high storage temperature. Also, PVP-iodine solutions (2% available iodine) have been stored at room temperature for one year. Two sets of solutions were made, one from PVP-iodine containing 10% available iodine and one from PVP-iodine powder containing 20% available iodine. In each case, the solutions lost less than 0.04% available iodine.

Irritation.—PVP-iodine has been found to be much less irritating and sensitizing to tissue and mucosa than other iodine preparations. When used in open wounds, the sensations reported range from "no sting" to "very slight sting." This work is reported by Shelanski (25) and Bogash (2).

Toxicity.—The toxicity of iodine is also reduced when it is in the form of PVP-iodine. This can be attributed to the fact that the iodine may not be present as such, at least not all at one time. This fact makes possible the safe usage of larger internal doses of iodine than previously possible. The toxicity of PVP-iodine as compared to other iodine is reported by Shelanski (25) and Bogash (2).

Preparation.—PVP-iodine powder can be prepared by mixing PVP powder with the required amount of crystalline iodine or by drying an aqueous solution of PVP and iodine. In the dry mixing method heat can be employed to facilitate the formation of a solution of iodine in the PVP. If the proper conditions are observed during the preparation, the resulting PVP-iodine will be stable in powder form and will produce stable aqueous solutions.

During the preparation of PVP-iodine powder, some of the available iodine is converted to iodide ion. It has been found that a 2:1 ratio of available iodine to iodide ion in the finished product is desirable.

ANALYSIS OF PVP-IODINE

Available Iodine.—A weighed sample is dissolved in about 100 ml. of distilled water and titrated with 0.1 *N* sodium thiosulfate using starch as indicator. For a high degree of accuracy and precision, the amount of sample used should be large enough to consume about 20 ml. of 0.1 *N* sodium thiosulfate. If the amount of available iodine is very small or if the starch end point is difficult to detect, then it is best to titrate the sample potentiometrically using a platinum *vs.* calomel electrode. Thiosulfate down to 0.0001 *N* can be used and an ordinary pH meter will serve as a potentiometer (use millivolt scale).

Iodide Ion.—A weighed sample is dissolved in about 100 ml. of distilled water and 1 *M* sodium bisulfite is added dropwise until the iodine color has completely disappeared. Then 50 ml. of 0.1 *N* silver nitrate is pipetted into the solution and 10 ml. of concentrated nitric acid is added. The excess silver nitrate is titrated with 0.1 *N* ammonium thiocyanate using a saturated solution of ferric alum as indicator. For a high degree of accuracy and precision the amount of sample used should be large enough to consume about 20 ml. of the silver nitrate. The % of iodide ion is obtained by subtracting the % of available iodine from the total iodine thus determined.

PVP Content via Kjeldahl Nitrogen.—Seventy to eighty milligrams of PVP-I is weighed in a 1-ml. beaker and placed in a Kjeldahl flask with 3.9 Gm. (±50 mg.) of nitrogen free potassium sulfate and 6 ml. concentrated sulfuric acid. The sample is digested at a vigorous boil until the solution is clear. If iodine remains in the neck of the flask, allow the solution to cool, add 2 ml. of water, and boil. The iodine escapes with the water vapor. This process should be repeated until all the iodine is removed. The flask is then cooled and about 15 ml. of water is added to dissolve the solids. The flask is placed on a Kjeldahl distillation apparatus, 24 ml. of alkali solution (50 Gm. NaOH and 5 Gm. $Na_2S_2O_3.5H_2O$ per 100 ml.) are added and the ammonia is distilled off in the usual manner. About 40 ml. of distillate is collected in 10 ml. of 2% boric acid contained in a 50-ml. Erlenmeyer flask. An additional 10 to 15 ml. of distillate should be collected in a second flask to check for complete removal of ammonia. The solutions are then titrated with 0.02 *N* H_2SO_4 using methyl purple indicator (Fisher Scientific Co.). A blank on the reagents is run in the identical manner.

H_2O in PVP-I.—Dissolve approximately 0.5 Gm. of PVP-I in 10 ml. *dry* N-methyl pyrrolidone.[1] Fill a sealed liquid cell (NaCl windows, 180 μ approx. spacer) with the solution. Run the infrared absorption spectrum of the solution from 2.00-3.40 μ. Water under these conditions exhibits an absorption maximum at 2.86 μ.

(i) Determine the absorptivity (a) of water in PVP-I/N-methyl pyrrolidone solution by calibration with known concentrations. (ii) Log % $T2.12/\%$ $T2.86$ = Absorbance (A) of H_2O + PVP-I + N-methyl pyrrolidone. (iii) Determine the absorbance of 0.500 Gm. PVP-I in 10 ml. N-methyl pyrrolidone at 2.86 μ as a background correction. $\therefore$ A − corr. × sample wt. PVP-I/0.500 = Abs. H_2O = $A_{corr.}$ (iv) $A_{corr.}/ab = C$ where b is cell thickness and C is concentration of H_2O in convenient units.

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[1] N-methyl pyrrolidone is obtainable from Antara Chemicals, 435 Hudson St., N. Y. The material is obtained dry by removing the lower boiling 20% via distillation (no great efficiency is needed). The next 60% of the material is distilled into a receiver protected from atmospheric moisture. The distilled solvent is stored in an ampul of the type used for acid chlorides and the neck of the ampul is sealed with a serum cap. Solvent is removed from the ampul using a hypodermic needle and syringe. This procedure prevents the methyl pyrrolidone from absorbing water from the atmosphere. An occasional IR check on the methyl pyrrolidone solvent should be made to insure its dryness.

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Notes

A Note on the Preparation of Fe^{59} Tagged Exsiccated Ferrous Sulfate*

By E. ROSEN and I. B. EISDORFER

EXTENSIVE CLINICAL DATA have been accumulated regarding the treatment of iron deficiency anemias with exsiccated ferrous sulfate, but with the advent of radioactive iron, the possibility of more definite clinical studies became apparent. However, this compound is not commercially available and its preparation has not been reported. A convenient, inexpensive procedure devised for converting Fe^{59} ferric chloride (which is the most readily available source) to Fe^{59} tagged exsiccated ferrous sulfate is described. Use of this method gives a resulting product which has a specific activity suitable for biological testing. The method of preparation is based upon assumptions generally considered to be true for radioactive substances: (*a*) that they undergo the same chemical reactions as unlabeled substances of the same chemical form; (*b*) that the participation of molecules is purely random; and, (*c*) that the system remains in steady state, except for the oxidation state of the iron.

EXPERIMENTAL

The following illustrates the reduction reaction described:

$$2\,Fe^{59+++} + SO_2 + 2H_2O \rightarrow 2\,Fe^{59++} + SO_4^{=} + 4H^+$$

The starting compound, Fe-59-P processed enriched in the form of $FeCl_3$ in HCl solution, was obtained from the Oak Ridge National Laboratory. To 32.7 ml. of a solution containing 1.53 ± 10% mc./ml. of Fe^{59} as $FeCl_3$ with a specific activity of 2633 mc./Gm., there was added 40 ml. of concentrated HCl and 328 ml. of distilled water. Sulfur dioxide was bubbled into this solution with agitation for 1 hour, at which time the solution was colorless and gave a negative test with KSCN test solution, indicating complete reduction of ferric iron. Two hundred grams of $FeSO_4{\cdot}7H_2O$ (reagent grade) was then added as a carrier, and the addition of SO_2 and agitation continued until solution was accomplished (approximately 1 hour). The addition of SO_2 was then stopped, and 1 liter of alcohol (S. D. #30) was added with agitation. Stirring was continued for 15 minutes and then the crystalline product was recovered by filtration on a Buchner funnel. The product was washed with 1 liter of alcohol in divided portions and dried at 50° at a pressure of 30 mm. of Hg for 12 hours. One hundred and twenty-four grams of a fine powder assaying 99.5% $FeSO_4{\cdot}1^1/_2H_2O$ was recovered. This represents 96% of the theoretical amount of $FeSO_4{\cdot}7H_2O$ present.

The millicurie strength of the material was determined by dividing the extrapolated net counting rate of a sample with the extrapolated net counting rate of a National Bureau of Standards Co^{60} *beta* ray standard, adjusted for decay and dilution according to standard procedure. The value determined was accepted as an approximation due to differences in maximum energy and energy distribution between the standard and the sample. The experimentally determined value of 16.2 mc. per 50 mg. of exsiccated ferrous sulfate is in good agreement with the 18.3 mc. value calculated to be the maximum theoretically possible.

* Received September 13, 1956, from the Smith, Kline and French Laboratories, Philadelphia, Pa.
The authors wish to express their gratitude to Dr. J. E. Christian for his advice and cooperation.

A Note on the Water Solubility of some N,N,-Dialkylamides*

By H. B. KOSTENBAUDER† and T. HIGUCHI

THE SOLUBILITY in water of some N,N-dialkylamides prepared for study as complexing agents (1) is quite high and suggests possible uses of the amides as a cosolvent for difficultly soluble pharmaceuticals in water. Since the possibility of preparing solutions of the amides in the concentrations reported by the authors in a previous publication (1) has been questioned by other workers (2), data are presented for establishing the identity and the water solubility of these N,N,N′,N′-tetramethylphthalamides.

Unsubstituted amides, with the exception of the low molecular weight aliphatic compounds, exhibit high melting points and relatively low water solubilities. These properties are generally attributed to the high degree of intermolecular association which occurs between the amide molecules as a result of hydrogen bonding. Substitution of alkyl groups for the acidic N-hydrogens in these compounds decreases the possibility of intermolecular association between the amide molecules, and favors solute-solvent interaction. Thus N,N,N′,N′-tetramethylphthalamide is soluble in water to the extent of 710 Gm. per liter at 30°, while phthalamide itself has a solubility of only 5.9 Gm. per liter at 30°.

A comparison of the physical constants of some N,N,N′,N′-tetramethylamides with those of some unsubstituted amides is presented in Table I. The preparation of the tetramethylamides and their tendency to form molecular complexes with certain pharmaceuticals were considered in a previous communication from these laboratories (1). The high water solubility and the complexing ability of amides of this type suggest that they might have important applications as industrial or pharmaceutical solvents.

TABLE I.—PHYSICAL CONSTANTS OF SOME N,N,N′,N′-TETRAMETHYLAMIDES

Amide	M. p.,[a] °C.	Water Solubility at 30°, Gm./L. Solution
Phthalamide	220	5.9
N,N,N′,N′-Tetramethylphthalamide	122–123	710
N,N,N′,N′-Tetramethylisophthalamide	137–138	676
N,N,N′,N′-Tetramethylterephthalamide	200–201	406
Succinamide	243	4.5 (15°)[b] 18 (50°)[c]
N,N,N′,N′-Tetramethylsuccinamide	83–86 80–81[d]	549

[a] Melting points uncorrected.
[b] "Handbook of Chemistry and Physics," 35th ed., Chemical Rubber Publishing Co., Cleveland, O., 1953, p. 1154.
[c] Seidell, A., "Solubilities of Organic Compounds," 3rd. ed., Vol. II, D. Van Nostrand and Co., Inc., New York, 1941, p. 260.
[d] Prelog, V., *Collection of Czechslov. Chem. Communs.*, 2 712(1930).

TABLE II.—CHARACTERIZATION OF N,N,N′,N′-TETRAMETHYLPHTHALAMIDES[a]

Compound		Calcd., %	Anal. Found, %	Infrared (Nujol)	Conductance Measurements
N,N,N′,N′-Tetramethylphthalamide ($C_{12}H_{16}O_2N_2$)	C	65.47	65.97	Agrees with structure	12,900 ohms (0.01 *M*)
	H	7.32	7.62		
	N	12.71	12.59	No OH or NH band; strong 1627 cm.$^{-1}$ for CO; 1596, 1576, 1502 cm.$^{-1}$ bands for CC of ϕ; 786, 751 cm.$^{-1}$ for *o*-disubstituted ϕ	89,500 ohms (water) 31,500 ohms (0.01 *M* sucrose)
				An apparent OH or NH band at 3430 cm.$^{-1}$ in $CHCl_3$, but not in CCl_4, suggests bonding with polar H of $CHCl_3$	79.5 ohms (0.01 *M* KCl)
N,N,N′,N′-Tetramethylisophthalamide ($C_{12}H_{16}O_2N_2$)	C	65.47	66.1, 66.7	Agrees with structure	14,600 ohms (0.01 *M*)
	H	7.32	7.71, 7.45		
	N	12.71	12.69	1630 cm.$^{-1}$ for CO; 1502, 1460 cm.$^{-1}$ for CC of ϕ	
N,N,N′,N′-Tetramethylterephthalamide	C	65.47	66.6, 66.0	Agrees with structure	40,000 ohms (0.01 *M*)
	H	7.32	7.21, 7.40, 7.01		
	N	12.71	13.12	1635 cm.$^{-1}$ for CO; 1527, 1483 cm.$^{-1}$ for CC of ϕ; 860 cm.$^{-1}$ for *p*-substituted ϕ	

The amides have no titratable groups in aqueous solution

[a] Elemental analyses, infrared analyses, and conductance measurements were furnished through the courtesy of the Research Division of The Upjohn Co., Kalamazoo, Mich.

* Received September 6, 1956, from the School of Pharmacy, University of Wisconsin, Madison.
† Present address: Temple University, Philadelphia, Pa.

Characterization of the Amides.—The results of infrared analyses, elemental analyses, conductance

measurements, and titration studies are in agreement with the structures proposed for the substituted phthalamides. These data are presented in Table II.

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A Note on the Effect of Hyaluronidase on Sulfathiazole Renal Toxicity*

By PRESTON T. FARISH and B. B. WILLIAMS

BUTT, HAUSER, AND SEIFTER (1) have demonstrated in clinical trials evidence of the protective action of the enzyme hyaluronidase against the formation of urinary calculi. This protection, they believe, is dependent on the coating, by hyaluronidase released colloidal material, of the crystal particles of substances undergoing saturation in the urine.

The protection thus provided against aggregation of crystals in urine by hyaluronidase has suggested the possibility of a similar effect in prevention of renal damage which often results from precipitation of crystals of sulfathiazole and acetylated sulfathiazole in kidney tubules.

TABLE I.—MORTALITY

Group	Subgroup	Sulfathiazole Gm./Kg./Day	Hyaluronidase TRU/Day	Mortality
I	1	0.4	5	0/6
	2	1.2	5	0/6
	3	2	5	1/6
	4	2.8	5	3/6
II	1	0.4	0	1/6
	2	1.2	0	0/6
	3	2	0	3/6
	4	2.8	0	4/6

TABLE II.—DEGREE OF KIDNEY DAMAGE

Group	Rat No.	Sulfathiazole Gm./Kg./Day	Hyaluronidase TRU/Day	Damage			
				Type I	Type II	Type III	Type IV
I	1	2	5	+	+	−	−
	2	2.8	5	−	+	−	−
	3	2.8	5	++	−	−	−
	4	2.8	5	+	++	++	+
	5	2.8	5	+	++	+++	+
II	1	2	0	++	−	++	+
	2	2	0	++	++	−	+
	3	2.8	0	+++	+	+++	−
	4	2.8	0	+	+++	+	+
	5	2.8	0	+++	−	−	−
	6	2.8	0	+++	++++	−	−
	7	2.8	0	+++	+++	++++	−

Sprague-Dawley rats ranging in weight from 70 to 140 Gm. were used in testing this hypothesis. Individually caged animals were divided into two groups of 24 animals per group (Groups I and II). Each group was divided into four subgroups of six animals each (subgroups 1, 2, 3, and 4) which received sulfathiazole at dose levels, respectively, of 0.4, 1.2, 2, and 2.8 Gm./Kg./day. The sulfathiazole was administered orally in 10 per cent acacia. In addition to sulfathiazole, group I rats received daily subcutaneous injections of hyaluronidase (Wydase®, Wyeth) in sterile physiological saline in doses of 5 TRU (turbidity-reducing units) per rat. To direct toxicity toward renal damage ammonium chloride was added to the drinking water to a concentration of 0.1 per cent, and intake was limited to 20 cc. per day for the first three days of the experiment, after which it was given *ad libitum*. Two rats similarly maintained as to caging, feed, and water served as blank controls. Administration of drugs was continued for thirteen days. Animals which died during the course of the experiment and those which were sacrificed at the termination were examined for gross signs of damage and kidneys were fixed in 20 per cent formalin for histopathology. Per cent mortality in groups is presented in Table I.

* Received August 22, 1956, from the School of Pharmacy, Alabama Polytechnic Institute, Auburn.
This project was supported by Alabama Polytechnic Institute Research Grant-in-Aid.

Although mortality in three of the four subgroups of group I was lower than that in the corresponding subgroups of group II, chi-square test did not show the difference to be significant at the 5 per cent level of probability. In further tests hyaluronidase dose was increased to 15 TRU. This increase failed to provide increased protection. This dose of hyaluronidase also failed to protect animals against acute toxicity following injection of single doses of sodium sulfathiazole by intraperitoneal route. Continuation of hyaluronidase administration for three days beyond cessation of sulfathiazole administration failed to reverse renal and urinary tract blockade produced in the rats at high dose levels of sulfathiazole.

In histopathology examination[1] the slides were given code numbers in order to avoid possibility of bias. Histopathological alteration that may be attributed to the effect of sulfathiazole was seen in the kidneys of twelve rats. Four types of histopathological alteration were seen. These were as follows:

Type I: Dilation of tubules in renal cortex.

Type II: Crystalline precipitate in the renal medulla.

Type III: Precipitate in the cortical tubules.

Type IV: Protein precipitate in the renal tubules.

The kidneys of animals showing some or all of these pathological alterations were compared and an estimate was made of the degree of each type of pathological alteration. In Table II is a listing of these arbitrarily assigned degrees of damage.

Although definite conclusions cannot be drawn from this preliminary study, it is believed that there is sufficient evidence of a protective effect of hyaluronidase against sulfathiazole-induced renal damage to warrant further investigation.

[1] The authors wish to acknowledge the contribution of Dr. G. R. Seibold of the Department of Pathology, Alabama Polytechnic Institute, in carrying out the histopathology study.

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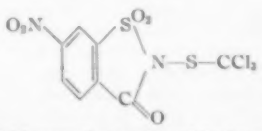
A Note on Fungicides: 2-Trichloromethanesulfenyl-6-nitrosaccharin and Related Compounds*

By GLENN H. HAMOR

COMPOUNDS containing the trichloromethanesulfenyl group have been shown to exhibit insecticidal and fungicidal activities (1, 2). One of these, N-trichloromethylmercapto-4-cyclohexene-1,-2-dicarboximide (captan), is widely used as a plant fungicide (3). Cohen has reported the synthesis and antifungal activity of 2-trichloromethanesulfenylsaccharin (4). Because 6-nitrosaccharin (5) was available from another study it was decided to prepare 2-trichloromethanesulfenyl-6-nitrosaccharin (I) for testing of antifungal activity.



I. 2-Trichloromethanesulfenyl-6-nitrosaccharin[1]

EXPERIMENTAL[2]

2-Trichloromethanesulfenyl-6-nitrosaccharin

The method of Kittleson (2) was used with some modification. Trichloromethanesulfenyl chloride[3] (5.1 Gm., 0.027 mole) was dissolved in 30 ml. of carbon tetrachloride and added dropwise with stirring during one hour at room temperature to a solution of sodium 6-nitrosaccharin (2.2 Gm., 0.009 mole) in 30 ml. of distilled water. The sodium 6-nitrosaccharin had been prepared according to the method of Noyes (7). Cooling the mixture for fifteen minutes in ice water and then filtering gave 2.3 Gm. (79%) of a gray-white solid melting at 140–145°. Recrystallization from acetone raised the melting point to 143–146°.

Anal.[4] Calcd. for $C_8H_3N_2O_5S_2Cl_3$: C, 25.51; H, 0.80; N, 7.44. Found: C, 25.69; H, 0.98; N, 7.52.

For purposes of comparison 2-methylsaccharin, synthesized according to the method of Merritt, Levey, and Cutter (6), was submitted for antifungal testing. Results obtained on these two compounds as well as that on N-methylphthalimide[5] are reported.

Antifungal Activity.[6]—The slide-dilution spore germination test was performed using *Stemphylium sarcinaeforme*, fourteen days old, and *Sclerotinia fruticola*, seven days old, as test organisms. Aliquots of each concentration of the test chemical in acetone in serial dilutions were placed in depression slide cavities. Two slides were prepared for each concentration for each test organism. The drops were dried, depositing the test compound as residues on the bottom of the cavities. Cavities were then filled with spore suspensions and incubated for seventeen hours. Results are expressed in Table I as per cent inhibition of germination at designated μg./cm.[2]

* Received September 24, 1956, from the School of Pharmacy, University of Southern California, Los Angeles.
This study was supported by the Pharmacy Alumni Association, University of Southern California.
The author is grateful to Dr. Norman Kharasch, Chemistry Department, University of Southern California, for suggestion of the problem.

[1] *Chemical Abstract* nomenclature, 2-(trichloromethylthio)-6-nitro-1,2-benzisothiazolin-3-one-1,1-dioxide.

[2] All melting points are uncorrected.

[3] Kindly supplied by the Stauffer Chemical Company.

[4] Analyses were carried out by the Microanalytical Laboratory, Chemistry Department, University of Southern California.

[5] Prepared by others.

[6] The author is indebted to Dr. James G. Horsfall, Connecticut Agricultural Experiment Station, New Haven, Conn., for performing the fungicidal testing as a screening agency for the Chemical-Biological Coordination Center, National Research Council, Washington, D. C.

TABLE I.—ANTIFUNGAL ACTIVITY EXPRESSED IN PER CENT INHIBITION OF SPORE GERMINATION

Compound	*Stemphylium sarcinaeforme* Concentration of compound in μg./cm.²				*Sclerotinia fruticola* Concentration of compound in μg./cm.²			
	1000	100	10	1	1000	100	10	1
2-Trichloromethanesulfenyl-6-nitrosaccharin	100	100	100	100	100	100	100	100
2-Methylsaccharin	100	100	54	36	100	100	100	100
N-Methylphthalimide	34	0	0	0	88	0	0	0

SUMMARY

1. 2-Trichloromethanesulfenyl-6-nitrosaccharin was synthesized and tested for antifungal activity.

2. It was found to be somewhat more active than 2-methylsaccharin in inhibition of spore germination of *Stemphylium sarcinaeforme* and *Sclerotinia fruticola* and much more active than N-methylphthalimide.

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A Note on a Simple Apparatus for Detecting Neurological Deficit in Rats and Mice*

By N. W. DUNHAM† and T. S. MIYA‡

NUMEROUS METHODS for detecting neurotoxicity are reported in the literature (1, 2), such as positional sense test, righting test, gait and stance test, muscle tone test, and equilibrium test. These tests may require an experienced observer, may not be as useful in rats as in mice, may not detect the very slight evidences of neurotoxicity, and/or may present a false positive test.

The rolling roller apparatus (Fig. 1) was devised while determining the neurotoxicities of a series of new synthetic anticonvulsants. This apparatus, in a large measure, overcomes the difficulties mentioned above. It has potential value for testing other types of pharmacologic agents such as skeletal muscle relaxants, convulsants, and central nervous system depressants.

The rolling roller apparatus employs a 115 v., 60 cycle, a.c., Bird Electric Kymograph, or a similar device, as a power source for turning the roller. The motor shaft is connected to one end of a 48 inch, $1^1/_8$ inch diameter roller from a window shade. The other end of the roller is trimmed to a suitable diameter and is inserted into a bearing. An inexpensive roller skate wheel was employed in this apparatus. The speed selector is set so that the roller makes 5 revolutions per minute. In order to perform multiple tests simultaneously, circular cardboard disks 12 inches in diameter are placed on the roller at suitable space intervals so as to divide the roller into equal compartments. The apparatus is held stationary by suitable clamps at a height of at least 15 inches above the table top. This height tends to discourage animals from jumping off the roller. The cages (see Fig. 1) serve to restrict the movements of the animals when they fall from the roller.

After trial doses are tested to approximate the TD-50 (Toxic Dose-50), three to four doses are administered to groups of 6 to 8 animals per dose. At predetermined peak times of drug activity, the animals are placed on the roller. A normal animal can maintain its equilibrium for an indefinite period of time on the revolving roller. One minute was found to be a suitable testing time. Neurological deficit is indicated by the inability of the animal to remain on the roller for the one minute test period.

The TD-50 is interpolated from a dose-response

* Received August 22, 1956, from the College of Pharmacy, University of Nebraska, Lincoln 8.

Abstracted in part from a thesis presented to the Graduate School of Purdue University by N. W. Dunham in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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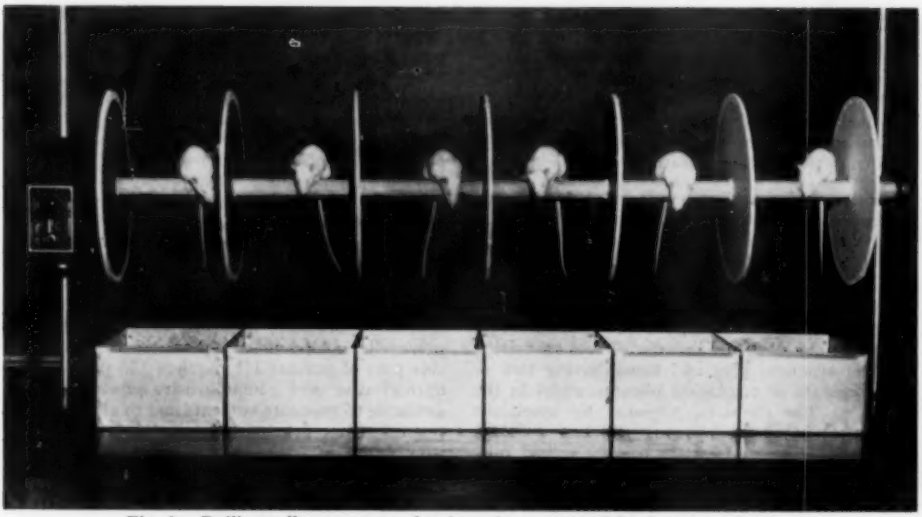

Fig. 1.—Rolling roller apparatus for detecting neurotoxicity in rats and mice.

curve constructed on semilogarithmic paper relating log dose and percentage exhibiting neurological deficit.

SUMMARY

A simple apparatus is described for detecting neurological deficit in rats and mice. It has potential value as a basis for comparing skeletal muscle relaxants, convulsants, and central nervous system depressants.

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Book Notices

Steric Effects in Organic Chemistry. Edited by MELVIN S. NEWMAN. John Wiley & Sons, Inc., New York, N. Y., 1956. vii + 710 pp. 15 x 23.5 cm. Price $12.50.

This book is a compilation of essays designed to elucidate the current knowledge of the influence of steric effects on the properties and reactivity of molecules. It does not attempt to serve as a textbook but rather presents the special information in the different contributors' fields. The subjects discussed are: conformational analysis; substitution at saturated carbon atoms; steric effects in aromatic substitution; additions to unsaturated functions; intramolecular rearrangements; olefin forming elimination reactions; cleavage reactions of the carbon-carbon bond; steric effects among the common organometallic compounds; steric effects on equilibrated systems; molecular complexes and molecular asymmetry; steric effects on certain physical properties; calculation of the magnitude of steric effects; and separation of polar, steric, and resonance effects in reactivity. The editor hopes that this book will convince practicing chemists of the laboratory usefulness of a knowledge of steric effects. Author and subject indexes are appended. Reference chemical libraries should include this book.

Pharmacognosy. 2nd ed. By ROBERTSON PRATT and HEBER W. YOUNGKEN, JR. J. B. Lippincott Company, Philadelphia, Pa., 1956. xii + 694 pp. 15.5 x 23.5 cm. Price $10.

The second edition of this book continues the modernization of the utilitarian approach in the presentation of the material in the field of pharmacognosy that was initiated in the first edition, which was reviewed in THIS JOURNAL **41**, 53(1952). Recent developments with drugs such as *Rauwolfia* and *Veratrum* species, new antibiotics, dextran, and plant and animal steroids in the syntheses of hormones are included in the scheme that combines the physiologic and chemical systems of drug classification. A new chapter on "Biosyntheses as the Origin of Drugs" has been included in the intro-

ductory part of the text, and an appended section lists poisonous plants and antidotes. A general index is included. The format, type, and illustrations are very good, with particularly sharp and clear structural formulas for organic compounds. The book is an excellent teaching text and can be used as a guide for laboratory work. It is recommended to teachers and librarians.

Chemistry of Carbon Compounds. Vol. IIIB. (Aromatic Compounds). Edited by E. H. RODD. D. Van Nostrand Company, Inc., New York, 1956. xviii + 1,669 pp. 15 x 23 cm. Price $25.

This book is the second part of volume III in a five-volume treatise on the chemistry of carbon compounds, and it completes the description of important classes of benzene derivatives containing a single aromatic ring and those having two or more separate or condensed benzene nuclei in the molecule. The chapters, prepared by specialists in the field, cover the following subjects: quinones and related compounds of the benzene series; phenolic monohydric alcohols, monoaldehydes, monoketones, and monocarboxylic acids; dihydric and polyhydric alcohols and their oxidation products with functional groups in separate side chains; phenyl derivatives of dihydric and polyhydric alcohols and their oxidation products; benzene derivatives with one or more unsaturated side chains; the phenylbenzene group; di-, tri-, and tetra-phenylmethanes; di-and poly-phenyl paraffins and their derivatives; monocyclic quasi-aromatic compounds; aromatic compounds with condensed nuclei: indene, napthalene and other bicyclic compounds; aromatic compounds with three condensed nuclei: anthracene, phenanthrene and related compounds; and aromatic compounds with condensed nuclei: fluorene, acenaphthene and polycyclic compounds. The extensive index for this part of volume III requires 135 pages. This authoritative and comprehensive work should be available to graduate students and to all who might require an excellent reference in this field of chemistry.

BOOKS RECEIVED

Encyclopedia of Chemical Technology. Vol. XV. (Waxes to Zymosterol and Index to Volumes 1–15). Edited by RAYMOND E. KIRK and DONALD F. OTHMER. Interscience Publishers, Inc., New York, 1956. xiv + 936 pp. 19.5 x 27 cm. Price $30. (Subscription Price $25).

Notes on Atomic Energy for Medical Officers. By the Staff of the Royal Naval Medical School, Alverstoke, Hampshire, England. Philosophical Library, Inc., New York, 1956. v + 169 pp. 12 x 18 cm. Price $4.75.

Heterocyclic Compounds. Vol. 5. Edited by ROBERT C. ELDERFIELD. John Wiley & Sons, Inc., New York, 1957. vi + 744 pp. 15.5 x 23.5 cm. Price $20.

Rauwolfia: Botany, Pharmacognosy, Chemistry and Pharmacology. By ROBERT E. WOODSON, JR., HEBER W. YOUNGKEN, EMIL SCHLITTLER, and JURG A. SCHNEIDER. Little, Brown and Company, Boston, 1957. xii + 149 pp. 16 × 24 cm. Price $5.50.

Pharmacognosy Laboratory Manual. By MAYNARD W. QUIMBY, RAYMOND W. VANDER WYK, and WILLIAM E. HASSAN, JR. Massachusetts College of Pharmacy, Boston, 1957. viii + 110 pp. 22 x 28.5 cm. Price $3.

Enzyme Antigen and Virus. By F. MACFARLANE BURNET. Cambridge University Press, New York, 1956. viii + 193 pp. 12.5 x 19 cm. Price $3.50.

Documentation in Action. Edited by JESSE H. ALLEN KENT, and JAMES W. PERRY. Reinhold Publishing Corporation, New York, 1956. xi + 471 pp. 16 x 24 cm. Price $10.

Psychiatric Research Reports of the American Psychiatric Association. No. 6. American Psychiatric Association, Washington, D. C., 1957. 211 pp. 15.5 x 23 cm. Price $2.

Rare Earths in Biochemical and Medical Research. (A Conference Sponsored by the Medical Division, Oak Ridge Institute of Nuclear Studies, October, 1955.) Edited by GRANVIL C KYKER and ELIZABETH B. ANDERSON. Office of Technical Services, Department of Commerce, Washington 25, D. C. xviii + 468 pp. 20.5 x 27 cm. Price $2.20.

Lectures in Immunochemistry. By Michael Heidelberger. Academic Press, New York, 1956. ix + 150 pp. 14.5 x 22 cm. Price $4.

Modern Methods of Microscopy. (A Series of Papers Reprinted from "Research"). Edited by A. E. J. VICKERS. Interscience Publishers, Inc., New York, 1956. 114 pp. 18.5 x 25 cm Price $3.50.

Calendar of the Pharmaceutical Society of Great Britain 1956–1957. The Pharmaceutical Press, 17 Bloomsbury Square, London, W. C. 1, England, 1956. 309 pp. 14.5 x 22 cm. Price 17s. 6d. (postage 10d.).

American Drug Index 1957. By CHARLES O. WILSON and TONY EVERETT JONES. J. B. Lippincott Company, Philadelphia, Pa., 1956. 650 pp. 14 x 21 cm. Price $5.

Technique of Organic Chemistry. Vol. III. 2nd ed. Part I: Separation and Purification. Edited by ARNOLD WEISSBERGER. Interscience Publishers, Inc., New York, 1956. ix + 873 pp. 16 x 23.5 cm. Price $17.50.

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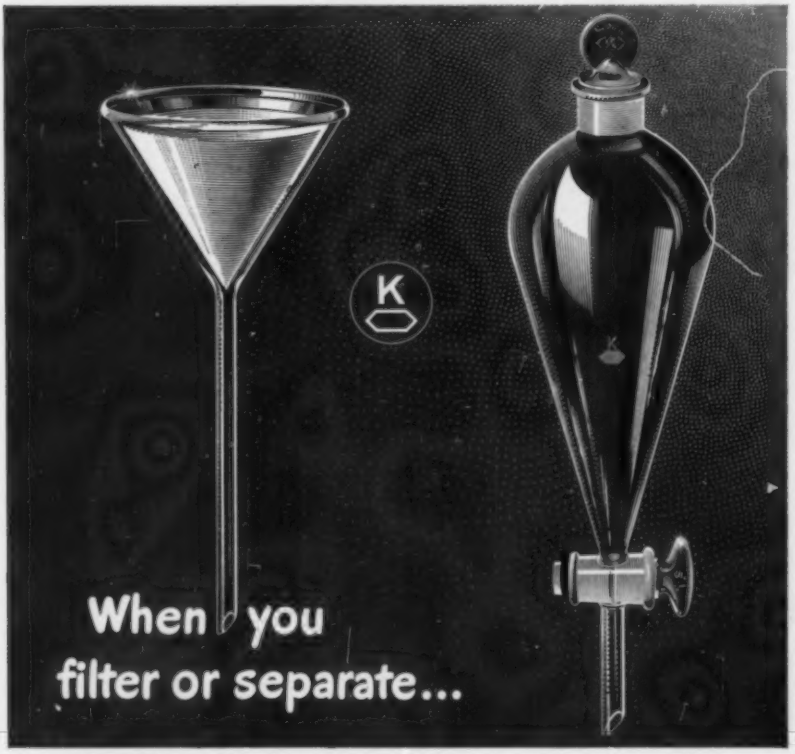

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They have 15% greater than nominal capacity which gives more shaking room for faster, more thorough mixing. Tight fitting stoppers and stopcocks prevent leaking during shaking. Larger neck openings make them easier to fill and clean. Taper of body has small inside diameter just above stopcock that permits accurate cut-off of liquid column . . . stem drains completely making quantitative separations easy and complete.

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Specify Kimble funnels when you order from your laboratory supply dealer. He'll be glad to give you information about prices, sizes and types. Or write Kimble Glass Company, subsidiary of Owens-Illinois, Toledo 1, Ohio.

KIMBLE LABORATORY GLASSWARE
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